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## FOREWORD

The first issue of a *British Journal of Pharmacology and Chemotherapy* is a notable event. It is not, of course, to be supposed that the growing volume and importance of pharmacological publication which have justified it, which, indeed, have created the need for it, represent a movement which is special to Britain, the phenomenon is to be seen throughout the scientific world. Pharmacology, we should recognize, has rapidly risen to major rank among the group of scientific disciplines which come within the scope of experimental medicine. This promotion has obviously been accelerated by the growing influence in practical therapeutics which Pharmacology has acquired through the recent development of its vigorous offspring, Chemotherapy—a fact fittingly recognized in the new Journal's title.

The event is one which naturally revives memories of the foundation, some 37 years ago, of the *Journal of Pharmacology and Experimental Therapeutics* by the late J. J. Abel, whose leadership and personal inspiration had already done so much to establish a vigorous school of Pharmacology in the United States of America. At that time, though Scotland had Chairs of Pharmacology at Edinburgh, Glasgow, Aberdeen and Dundee, with distinguished incumbents, England had so far, recognized a need for only one full-time Chair, which had been created four years earlier at University College, London. A. R. Cushny, who had been a pupil of Cash at Aberdeen, and later of Schmiedeberg, and had been Abel's successor at Ann Arbor, Michigan, had come back in 1905 to be the first holder of the new Chair at University College. W. E. Dixon, though still holding a position of minor academic rank, was then already creating a centre of lively interest and experimental activity in Pharmacology at Cambridge, and R. B. Wilde, though largely occupied in clinical work, was giving a regular course in Pharmacology from a Chair in Liverpool. For the rest of England, those who found their way into Pharmacology had done so largely by natural interest and their own unguided exploration.

To all these the launching of the *Journal of Pharmacology* by Abel had provided a much-needed outlet for pharmacological papers in the English

language The earlier volumes of that Journal bear witness to the fact that, from the outset, it offered effective hospitality to papers by British workers Their opportunities of publication had otherwise been almost limited to the weekly medical journals and the *Journal of Physiology*, and the editor of the latter, the late J N Langley, was showing a steadily increasing reluctance to accept papers which could be regarded as pharmacological I well remember going in 1911 to consult Cushny about a difficulty created for me by Langley's refusal of a paper of mine, for the reason that Pharmacology was encroaching unduly on his space I suggested to Cushny that, while we were all grateful to Abel's Journal for what it was already doing for us, we should feel happier if, though still published at Baltimore, it could be recognized as having a wider, English-speaking interest and editorial responsibility The upshot was a friendly negotiation leading to a joint editorial control of the Journal by Abel and Cushny, assisted by advisers of whom several were now to be British The arrangement lasted in that form till Cushny's premature death in 1926, and then, with successors in due course to Abel as well as to Cushny, till the present time It has served British Pharmacology well and we have abundant reason to be grateful to our American colleagues for these 35 years of association and shared responsibility We may regard it, perhaps, as an early, spontaneous and limited example of that wider collaboration between the scientists of the English-speaking nations which in the recent war became so intimate and so efficiently organized

On the maintenance of that full and friendly collaboration in science much may depend for the future of the world, and we must hope to make it even stronger and closer in Pharmacology than it has been On the other hand, it is necessary to watch developments which the demands of war itself have accelerated, and to recognize the likelihood of a rapidly and healthily growing demand for space to publish papers dealing with Pharmacology and Chemotherapy On both sides of the Atlantic the intensive and organized researches of the war period must have caused a serious accumulation of such matter Pharmacologists, too, of countries which have suffered aggression and spoliation in war may well be seeking opportunity for publication, of which their own countries cannot yet offer a prospect It would not be fair to expect a single journal to cope with these heavy arrears, as well as with the expanded output which the wartime development is certain to leave as a lasting consequence, and at the same time, to ensure promptitude of publication for important new discoveries in this widening field, from Britain and British Dominions as well as from the United States of America The British Pharmacological Society, which had come into existence long after Abel's *Journal of Pharmacology* was founded, accordingly reviewed the position and came to the conclusion that they could best serve the common interest, and best show their lasting gratitude for the friendly help and collaboration they have received from their American colleagues, by starting a separate Journal edited and published in Britain, and thus lightening

the prospective load on the one in which they have, for so many years, been generously allowed to have a share of interest and control. The British Medical Association has offered to sponsor the new *British Journal of Pharmacology* and to be responsible for the technical aspects of its publication, leaving the editorial responsibility entirely in the hands of a Board appointed by the British Pharmacological Society. It is certainly the hope of everybody concerned with the venture, that there will still be abundant opportunities of co-operation and friendly interchange between the new *British Journal* and the *Journal of Pharmacology and Experimental Therapeutics*, which has so long and so well served British as well as American achievements in this field.

March 12, 1946

H H D

## ANALGESIC ACTION OF PETHIDINE DERIVATIVES AND RELATED COMPOUNDS

BY

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(Received August 17 1945)

Comprehensive and up-to-date reviews on the actions and evaluation of analgesics have been published in America by Small, Eddy, Mosettig and Hummelsbach (1938), and by the Committee on Drug Addiction under the chairmanship of White (1941). Fourneau (1938) has discussed much of the older work on the relationship of chemical constitution to analgesic efficiency. Schaumann (1940) has reported on the synthetic compounds derived from 4-phenyl-piperidines synthesized by Eisleb (1941), and by his analysis has illuminated our conceptions of the structural essentials for analgesic activity. He concluded that of the compounds he examined an optimum was reached in ethyl-4-phenyl-1-methyl-piperidine-4-carboxylate hydrochloride (pethidine, demerol, dolantin)—a compound which has received the recognition of an approved name, pethidine hydrochloride, in the 7th Addendum to the *British Pharmacopoeia* (1944). Woolfe and Macdonald (1944) have attempted an evaluation of the analgesic activity of this drug, using a simple technique for the measurement of such action in mice. Pethidine already has an extensive clinical literature, and there is reasonable agreement between estimates of its efficiency, relative to other drugs, on mice and men.

This paper describes the application of the experimental technique to a large number of pethidine derivatives and related compounds, most of which have been synthesized for the first time by Bergel *et al* (1944). Very few of these have been investigated by Schaumann. While no immediate claim for clinical importance can be made for any of the active compounds, except perhaps for *iso*-pethidine (C 21) (cf Glazebrook and Branwood, 1945), our pharmacological experience

with them adds to the confidence with which one can attempt to relate constitution to analgesic activity

## EXPERIMENTAL

In our studies of these drugs, analgesic activity was assessed by a hot plate technique (see Woolfe and Macdonald 1944). The mouse under observation was placed on a smooth metal surface kept at 55° C for 30 secs, or less if it appeared to suffer discomfort as indicated by movements of the hind limbs during such exposure. In the absence of obvious hind-limb movement, it was assumed that analgesia had been obtained.

All drugs were given hypodermically, and all were tested against one sample of pethidine hydrochloride to determine relative activity, using mice from one batch, of approximately the same age and weight, in each comparison. The compounds were usually supplied and tested as hydrochlorides, but some were bases and were dissolved in a minimum of hydrochloric acid. A few other salts were occasionally used and such are indicated in the tables. None was written off as inactive on fewer than eight mice, and all showing analgesic activity were tested on not less than thirty animals. For some, over one hundred mice were used. The figure given for the relative activity was obtained from the comparison of the weights of the drugs, calculated as base, giving freedom from obvious discomfort lasting for 30 mins. An illustrative experiment on the stereoisomers of nor-*iso*-pethidine is summarized in the protocol.

PROTOCOL NOR-*ISO*-PETHIDINE ISOMERS

(In this batch of mice, the figure for the dose of pethidine base required to produce analgesia for 30 min was 30 mg/kg.)

Optical Form	Dose (mg./kg.)	Proportion of Animals in which Analgesia occurred	Average Duration of Analgesia (A)	Standard Deviation of (A)	Dose to produce Analgesia lasting 30 min	Analgesic Activity (Pethidine = 1)
dl C. 23	50	5/10	17.6	8.0	123	1/4
	100	7/10	28.9	8.8		
	150	7/10	31.6	9.7		
l C. 24	30.4	3/10	—	—	58	1/2
	45.6	5/10	26.4	9.9		
	60.8	6/10	30.9	13.8		
	76.0	6/10	36.8	15.1		
	91.2	7/10	40.3	14.7		
d C. 25	60.8	0/10	—	—	—	0
	91.2	2/10	Convulsions in 1/10	—		
	121.6	4/10	Convulsions in 5/10	—		

## RESULTS

(1) *Certain Salts of Pethidine* (Table I)—It will be seen that the hydrochloride is reasonably stable for six months in aqueous solution (C 1a). The hydriodide (C 2) is equally effective, though less soluble.

TABLE I

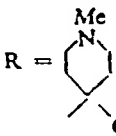
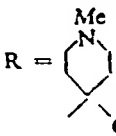
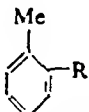
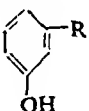
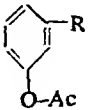
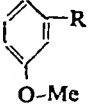
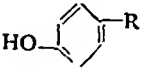
Compound	Name	Relative Activity (Pethidine = 1)	Remarks
C 1	Pethidine hydrochloride (Formula II, p 12)	1	LD <sub>50</sub> 250 mg./kg
C 1a	The same after 6 months in solution in ampoules	1	Reasonably stable
C 2	Pethidine hydriodide	1	„ „

(2) *Variations in the Phenyl Ring of Pethidine* (Table II)—Our observations on over 150 animals indicated that ethyl 4-(*o*-tolyl)-1-methylpiperidine-4-carboxylate or 2'-methyl-pethidine, as hydrochloride (C 3), is a more potent and longer-lasting analgesic than pethidine itself, although as hydriodide or tartrate (C 4, C 5) its activity is of the order of that of pethidine hydrochloride.

Ethyl 4-(3'-hydroxyphenyl)-1-methylpiperidine-4-carboxylate or 3'-hydroxy-pethidine (C 6) we found substantially equal to pethidine itself. Constitutionally, it is nearer to morphine. The corresponding acetoxy-compound (C 7) also had activity of the same order. This is in agreement with Fourneau's view of the uselessness of acetylation of the phenolic hydroxyl. The methoxy- (C 8) had less activity than the hydroxy-compound (C 6), which shows a relationship similar to that of codeine to morphine and heroin. The greater stability of the methoxy-group to hydrolysis *in vivo* as compared with that of the acetyl-group would account for this phenomenon. Schaumann reported that the corresponding 4'-hydroxy-compound (C 9) had only one-fifth of the activity of pethidine, which we have confirmed. We did not examine other 4'-substituted derivatives.

(3) *Variations in the Ester Group* (Table III)—It is clear that the alcohol component of the ester group profoundly affects activity. In sub-lethal doses, the methyl- (C 11), butyl-, cyclohexyl- and glycol-esters and the amides showed no significant analgesic activity. Some of these observations confirm those of Schaumann (1940). All deviations from the ethyl-ester group which we have tried resulted in considerable loss of analgesic value. Moderate activity is retained in the *iso*-propyl- (C 12) and allyl-ester (C 13), but even less in the *n*-propyl-ester (C 14). Although the ketones (C 15, 16, 17), as shown by Schaumann, are quite active, the secondary alcohol (C 18) obtained by reduction of the methyl ketone (C 15) and its O-acetyl derivative showed no analgesic action in doses up to 80 mg/kg.

TABLE II

<div style="text-align: center;">  <p>R =  P = Pethidine</p> </div>				
Compound No	Formula	Name	Relative Activity (Pethidine = 1)	Remarks
C 3	<div style="text-align: center;">  <p>HCl</p> </div>	2'-methyl-P	1½	Rapidly loses activity on standing at room temperature at pH 5.4 LD <sub>50</sub> = 200 mg./kg
C 4	Same, as hydriodide		1	Retains activity for 5 days + in solution In higher doses shows more prolonged action than corresponding doses of pethidine (C 1)
C 5	Same, as tartrate		1	
C 6	<div style="text-align: center;">  </div>	3'-hydroxy-P	1	
C 7	<div style="text-align: center;">  </div>	3-acetoxy-P	1	
C 8	<div style="text-align: center;">  </div>	3-methoxy-P	1.2	
C 9	<div style="text-align: center;">  </div>	4-hydroxy-P	1.5	(Confirms Schaumann)

(4) *Combination of Features of 2 and 3* (Table IV)—The lactone of 4-(2'-hydroxyphenyl)-1-methylpiperidine-4-carboxylic acid (C 19) acts weakly. The corresponding 3'-methoxy-lactone (C 19a) in doses up to 120 mg/kg and the related 2-methyl-3-4'-spiro-(1'-methylpiperidine)-coumaran (C 20) up to 80 mg/kg showed no analgesic activity, in spite of the fact that they possess additional features of the morphine nucleus.



TABLE III

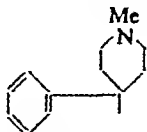
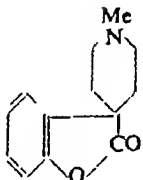
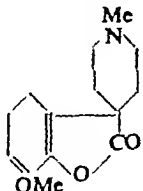
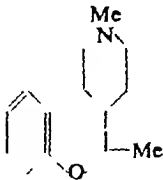
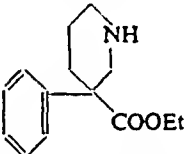
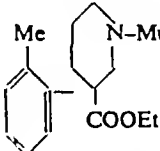
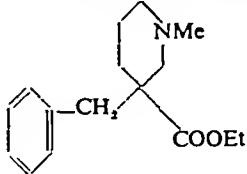
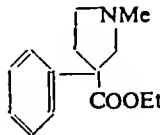
<div style="text-align: center;"> <math>R' = </math>  </div>			
Compound No	Formula	Relative Activity (Pethidine = 1)	Remarks
C 10	$R' CO_2H$	0	Tested at 160 mg./kg. Depressant Higher doses produce great depression, including that of respiration
C 11	$R' CO_2Me$	$< 1/6$	
C 12	$R' CO_2CHMe_2$	$1/2$	
C 13	$R' CO_2CH_2CH_2CH_3$	$1/2$	Extreme depression
C 14	$R' CO_2Pr$	$1/3$	
C 15	$R' CO Me$	$1/3$	
C 16	$R' CO Et$	$1/3$	Excitement followed by depression Tested at 80 mg./kg
C 17	$R' CO Pr$	$1/2$	
C 18	$R' CHOH Me$ (and $R' CH(OAc)Me$ )	0	

TABLE IV

Compound No	Formula	Relative Activity (Pethidine = 1)	Remarks
C 19		$1/4$	Active only in rather toxic doses
C 19a		0	Tested at 120 mg/kg
C 20		0	Tested at 80 mg/kg.

(5) *3-Phenyl-piperidine or iso-pethidine Series* (Table V)—Ethyl-3-phenyl-1-methylpiperidine-3-carboxylate or *iso-pethidine* (C 21) showed pronounced and prolonged though somewhat irregular activity. As all these compounds were synthesized as racemates, it seemed desirable to investigate some of the pure optical isomers. In the case of *nor-iso-pethidine* (C 22), it seems that pharmaco-

TABLE V

Compound No	Formula	Relative Activity (Pethidine = 1)	Remarks
C 21	<i>Iso-pethidine</i> as hydrochloride (Formula III p 12)	1/2	Irregular, long lasting in some, little or none in others
C 21a	Same, as tartrate	1/2	
C 21b	Tartrate after four months' storage in solution	1/2	
C 22	 <chem>CCOC(=O)C1CCNCC1c2ccccc2</chem> <i>dl-nor-iso-pethidine</i> in hydrochloric acid solution	1/4	Rather irregular and rather toxic
C 23	Same, in tartaric acid solution	1/4	" "
C 24	<i>l-nor-iso-pethidine</i> acid tartrate	1/2	Lethal at 200 mg./kg
C 25	<i>d-nor-iso-pethidine</i> acid tartrate	Nil	Convulsions at 300 mg./kg
C 26	 <chem>CCOC(=O)C1CCN(C)CC1C2=CC=CC=C2C</chem> <i>2-methyl-iso-pethidine</i> hydrochloride	3/4	Action delayed but prolonged
C 26a	Same, as hydriodide mixed with pethidine HCl, 25 mg./kg.	1	Reliable and prolonged action
C 27	 <chem>CCOC(=O)C1CCN(C)CC1Cc2ccccc2</chem>	0	Tested at 120 mg./kg.
C 28	 <chem>CCOC(=O)C1CCN(C)CC1c2ccccc2</chem>	0	Tested at 200 mg./kg.

logical activity is, as usual, found in the laevo form (C 24) and the dextro-form (C 25) is practically inactive

In this as in the pethidine series activity appears to be enhanced by introducing a 2'-methyl group into the phenyl ring (C 26) The specificity of the ethyl-ester group is here even more pronounced, as the methyl- *n*-propyl- and *isopropyl*-

TABLE VI

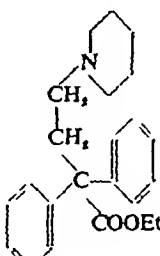
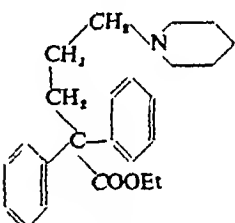
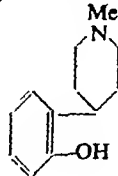
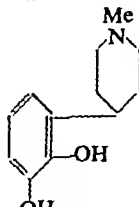
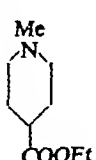

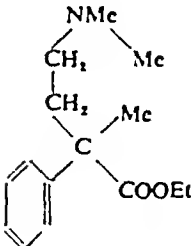
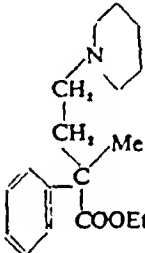
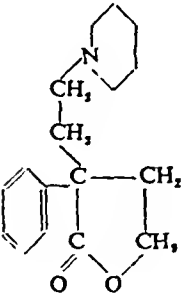
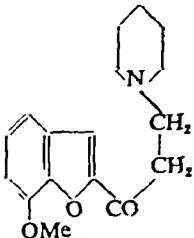
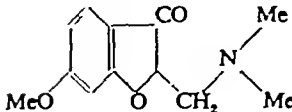
Compound No	Formula	Relative Activity (Pethidine = 1)	Remarks
C 29		1/2	Wide side actions
C 29a		1/6	Strongly depressant
C 30		0	Tested at 120 mg./kg.
C 31		0	Tested at 120 mg./kg.
C 32		0	Tested at 120 mg./kg.

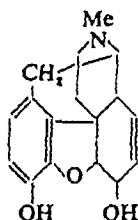
TABLE VI—*continued*

C 33		0	Tested at 120 mg/kg
C 34		1/6	Very irregular
C 35		0	Tested at 120 mg/kg.
C 36		0	Tested at 120 mg/kg.
C 37		0	Tested up to 125 mg/kg. by Hoffmann-La Roche Laboratories, Basle, on the guinea pig
C 38		0	Tested up to 125 mg/kg. by Hoffmann-La Roche Laboratories, Basle, on the guinea pig

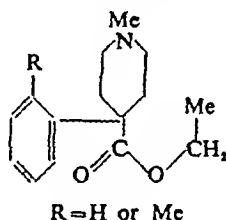
esters have no significant analgesic action in doses up to 120 mg/kg. Replacement of the phenyl- by a benzyl-group (C 27) or of the piperidine ring by a pyrrolidine ring (C 28) also led to complete loss of analgesic activity.

(6) *Miscellaneous Compounds* (Table VI)—Systematic testing of compounds C 29–C 36 revealed activity only in the compound C 29, thus confirming previous observations. The homologous compound C 29a, which bears the same relation to C 29 in respect to the position of the N-atom as *iso*-pethidine does to pethidine, showed only slight activity. Complete removal of either the ester- or the phenyl-group (C 30–C 33) resulted in total loss of activity in doses up to 120 mg/kg body weight. Eisleb (1942) similarly found that 4-phenyl-1-methylpiperidine was inactive. In compounds C 34 and C 35, in which the original piperidine ring of pethidine has been opened, very slight or no analgesic action was observed. More drastic alterations in the structure as in C 36, 37, 38 (the last two tested by the Research Laboratories of Messrs. Hoffmann-La Roche, Basle) led again to loss of analgesic activity.

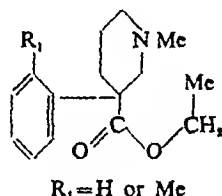
### DISCUSSION



Morphine  
I



Pethidine series  
II



*Iso* pethidine series  
III

Schaumann (1940) drew attention to the structural similarity of pethidine to morphine, though the synthetic drug lacks a number of the features of the natural product. His emphasis on the unimportance of the phenanthrene skeleton may, in our opinion, be exaggerated, especially since the 2'-methyl-pethidine (C 3, 4 and 5) showed in our experiments an increase in potency which may be related to its closer approximation in shape to the phenanthrene feature of morphine (cf. I and II (R = Me)).

It is interesting to find that 3'-hydroxy-pethidine (C 6) is not less active than pethidine, as Schaumann found to be the case with the 4'-hydroxy compound (C 9). The loss of activity following methylation (C 8), but not acetylation (C 7), of 3'-hydroxy-pethidine, may be due to the same causes as the similar loss of activity following methylation of the phenolic hydroxyl of morphine.

How can we account for the relative specificity of the ethyl-ester? Apart from the *iso*-propyl- (C 12), allyl- (C 13) and, to a lesser degree, propyl-esters (C 14), all other esters show no or insignificant activity, so, according to

Schaumann do the amides Ketones on the other hand, are active, but here the length of the ketone chain seems less critical than that of the ester group It is worth while mentioning here that Jensen *et al* (1943) found that acyl derivatives of 4-phenyl-4-hydroxyl-1-methyl-piperidine have considerable activity, the maximum of which is possessed by the propionic acid derivative Compounds C 19, an *iso*-coumaranone derivative C 19a, its 7-methoxy-homologue, and C 20 a 2-methyl-coumaran derivative show hardly any activity, although their structure bears a greater resemblance to the benzofuran feature of morphine than any compounds of the true pethidine series

Accepting Schaumann's hypothesis, which is supported by our own results that the phenyl-piperidine structure is essential for the optimal activity in this group of synthetic analgesics, we should now like to speculate on other aspects of the problem

(1) As to the hydroaromatic rings in the morphine molecule, the one carrying the secondary alcohol group and the double bond is replaceable by an open chain consisting either of an esterified 4-carboxyl-group, a 4-acyl-group or a 4-hydroxy-acyl derivative The fact that there exists an optimal length of the open chain for maximum analgesic activity, which is identical in each case and consists of 4 atoms, either carbon or carbon and oxygen, leaves little doubt as to the part played by these groupings in "mimicking" this hydroaromatic ring The inactivity of the secondary alcohols corresponding to the ketones suggests that the carbonyl groups in these chains may subserve a function similar to that of the oxygen bridge in the morphine molecule

(2) In the nitrogen ring system, the change from the 4-phenyl- (II) to 3-phenyl-piperidine (III) does not greatly reduce analgesic power Indeed, some of these *iso*-compounds, though somewhat irregular in their effects, have produced analgesia of long duration Again, the specificity of the ethyl ester group is dominant

In the case of compound C 22, we have demonstrated that pharmacological action is associated, as is usual, with the 1-form, but many more quantitative and comparative assays are required before conclusions can safely be drawn

(3) If the piperidine ring of the pethidine series is opened, serious loss of activity occurs (C 34 and 35) But if a second phenyl-group is introduced into such a system as in C 29, analgesic power is restored to a certain extent Recent reports, moreover (cf Report on Pharmaceuticals I G Farbenindustrie, 1945), indicate that replacement of the carbethoxy-group in such substances by appropriate acyl-groups leads to compounds of considerable activity However, in the absence of full data it would be futile to speculate on the relationship between such compounds and those of the pethidine or *iso*-pethidine series

(4) The results obtained in this study seem to indicate that the shape or fit of the molecule as a whole is more important in determining its analgesic value than any precise duplication of any one fraction of the morphine structure

## SUMMARY

(1) Some 50 synthetic compounds of the pethidine type were examined for analgesic activity using mice and a hot-plate technique

(2) Some of these appear to have a stronger action than pethidine—notably the 2'-methyl-pethidine, and some have a longer action—notably the laevo-isomers of 3-phenyl-piperidine or iso-pethidine derivatives

(3) Many inactive compounds were tested and some "rules" governing loss of potency are suggested

(4) The nearer the compound approaches to morphine in the shape of its molecule, the more likely it is to be a good analgesic

(5) Extensions of this work are planned in the hope that facts will emerge which will provide for a maturer judgment on the relationship of chemical constitution to analgesic value

We gratefully acknowledge help from Drs J W Haworth and N C Hundley and Miss M Koenigstein, who have prepared some of the compounds used in this work

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## CHEMOTHERAPEUTIC ACTION OF DYES IN TYPHUS INFECTION OF MICE

By

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(Received November 1 1945)

Several papers published during 1944 revealed that the rickettsiae of typhus fever are susceptible to attack by chemotherapeutic agents. Such activity has been shown in experimentally infected mice by Andrewes, King, van den Ende and Walker (1944), using *p*-sulphonamidobenzamidine (V147) and the corresponding amidoxime (V 186) by Yeomans, Snyder, Murray, Zarafonitis and Ecker (1944), using *p*-aminobenzoic acid, by Moragues, Pinkerton and Greiff (1944) with penicillin, by Peterson (1944) with forbisen and toluidine-blue and by Kikuth and Schilling (1944) with methylene-blue. No good results in experimental animals, other than mice, have been reported. The compounds, V147 and V186 and penicillin have proved unpromising in human typhus (van den Ende, Stuart-Harris, Fulton and Niven, 1946).

This paper records the results of extending Peterson's observations on toluidine-blue by studying the action of other dyes, particularly those chemically related to it.

### TECHNIQUE

#### STUDIES OF *in vivo* ACTION IN MICE

Most of the workers, other than ourselves, mentioned above, have used the intraperitoneal route for infecting the mice in their studies. This has the disadvantage that it is available only for murine typhus, not for epidemic strains, nor is it so well adapted for quantitative comparison of drugs as the intranasal technique we employed. This method we have continued to use. Suspensions of rickettsiae, kept at  $-76^{\circ}\text{C}$ , will maintain their potency unchanged for many months, and suitable dilutions inoculated intranasally into mice will produce isolated foci on the lungs. The foci can be counted with satisfactory reproducible results, and the reduction in count used to judge the effect of drugs. Groups of six mice were used for each drug, with an appropriate control group of six mice. Details are given in the earlier paper (Andrewes *et al* 1944). All intranasal inoculations were carried out in the special inoculation box (van den Ende, 1943), without the aid of which, we are convinced, the intranasal technique will almost certainly lead to accidental laboratory infections.

In routine testing of many chemotherapeutic agents over two years ago we failed to detect any activity on the part of toluidine-blue or methylene-blue. This was doubtless



because these drugs are very poorly tolerated by the intraperitoneal route which we were using. For further work with dyes we used one of three methods

(a) Drugs were mixed in a known percentage with the moist food mash. This method was labour-saving, but did not readily disclose the actual amount of drug taken, particularly as sick mice ate poorly

(b) Drugs were administered into the oesophagus by means of a 1 cc syringe and wide needle, of which the point had been cut off and the end blunted. This was done, as in our earlier intraperitoneal tests, two hours before infection, twice daily on the two following days, and on the morning of the third following day

(c) The drugs were given subcutaneously according to the same time schedule as under (b). Many caused local oedematous reactions and consequently each of the six injections was made at a different site

#### STUDIES OF *in vitro* ACTION AND TESTS ON RABBITS SKINS

Peterson reported that toluidine-blue inactivated murine typhus rickettsiae *in vitro*. We accordingly included in our studies tests of such action. Suspensions of murine rickettsiae were held in contact with aqueous solutions of various dyes for 45 minutes at room temperature, and then inoculated in 0.1 cc quantities intradermally into skins of shaved rabbits. Several dilutions of each of four or more dyes could be tested on the skin of one rabbit. Staining or inflammatory reactions caused by the dyes themselves rarely caused trouble in the dilute solutions in which we used them. Specific raised erythematous lesions, such as have been described by Giroud (1938), appeared within two or three days whenever the rickettsiae had not been inactivated; they reached their maximal intensity after about five days. Since the texture of the rabbits' skin may affect the size and nature of a rickettsial lesion, several control inocula of rickettsiae alone were always made in different positions into the animal's skin. The number of rickettsiae in the inoculum did not materially affect the results of drug tests over a wide range (1/100 to 1/10,000 of stock suspension), so we used throughout a 1/100 dilution of a stock suspension. 0.05 cc of which, when diluted 1/10<sup>3</sup>, was capable of producing 40 lesions in a mouse's lungs. The time of contact of rickettsiae and dye did not greatly affect the issue, though slightly better inactivation occurred when contact was for one hour at 37°C. Very probably some action of dyes on the organisms continued after the mixture had been injected intradermally.

#### RESULTS

*Mouse Tests*—We readily confirmed Peterson's finding that toluidine-blue had a chemotherapeutic action when given mixed with the food. Table I shows that it wholly suppressed lung lesions when given in a 1.5 per cent concentration, that 0.5 per cent was less effective and 0.1 per cent useless. Oral administration with a syringe suppressed lesions wholly when 5 mg were given on six occasions. Results were less striking when the dose was reduced. The drug given subcutaneously in doses of  $6 \times 1$  mg or  $3 \times 2$  mg was also completely effective; these doses, however, produced considerable oedematous swellings at the sites of inoculation. We confirmed our earlier findings that toluidine-blue was ineffective when given in the maximal dose which was tolerated intraperitoneally (1 mg).

Table I shows that methylene-blue was equally effective. Possibly it was a little more so, but the differences were not significant. Similar results were obtained in a feeding test with medicinally pure zinc-free methylene-blue. These experiments were carried out with murine typhus (Wilmington strain). In two

experiments toluidine-blue was shown to act also in epidemic typhus infection, though rather less effectively. Six doses of 5 mg *per os* reduced the lung spot-count from 56 to 14.

Of other dyes tested only three (selenium-methylene-blue, new methylene-blue and 3-diethylamino-7-di-*n*-butylaminophenazthionium chloride) had any

TABLE I

CHEMOTHERAPEUTIC EFFECT OF METHYLENE BLUE ANALOGUES AGAINST MURINE TYPHUS IN MICE

Drug	How given	Dose	Lung Spot Count (average of 6 mice)	Lung Spot Count in un inoculated mice (average of 6)
Toluidine-blue	Mixed with food	1.5%	0	34
"	"	0.5%	2	44
"	"	0.5%	4.5	28
"	"	0.1%	28	28
Toluidine-blue	<i>Per os</i> (syringe) twice daily (6 doses in all)	5 mg	0	42
"	"	2.5 mg	1.5	42
"	"	2 mg.	13	47
"	<i>Per os</i> (syringe) once daily (3 doses in all)	5 mg	2	27
Toluidine-blue	Subcut 6 doses	1 mg	0	24
"	" 3 "	2 mg	0	27
Methylene-blue	Mixed with food	1.5%	0	44
"	"	0.5%	0	28
"	"	0.1%	10	28
"	"	0.1%	17	24
Methylene-blue	<i>Per os</i> (syringe) (6 doses in all)	2 mg.	12	47
Methylene-blue	Subcut 6 doses	1 mg	0	24
"	" 3 "	0.5 mg	1	30
New-methylene-blue	Subcut 6 doses	1 mg	2	45
3-Diethylamino-7-di- <i>n</i> - butylaminophenaz- thionium chloride hydrochloride	Subcut 3 doses	1 mg	0	12
"	" 3 "	0.25 mg	20	30
Selenium-methylene- blue	Subcut 3 doses	2 mg	3.5	30
"	" 3 "	0.5 mg.	14	30

action at all *in vivo*. The first two of these were apparently slightly less active than, the third about equal to methylene and toluidine-blues. With the other dyes, no significant differences occurred between the spot-counts of treated and control mice. They were given at their maximal tolerated dose as shown in

TABLE II  
DETAILS OF TESTS OF DYES AGAINST TYPHUS IN RABBITS AND MICE

Group to which dye belongs	Dye	Route of inoculation and dose (m.t.d.) used for mouse tests—in mg. unless otherwise stated	Activity in mouse test (see Table I)	Effective dilution <i>in vitro</i> as shown by rabbit test
Acridines	Proflavine Acridiflavine 2 7-Diaminoacridine 2 8-Tetramethyldiamino-10-methyl-acridinium methosulphate Atebrin	0.37 i.p. 0.06 i.p. 8 i.p. no test 2 s.c., also tested 0.5%	0	Ineffective at 1:2,000
Oxazines	Brilliant-cresyl-blue Cresyl-fast-violet 2B Capri-blue G O N Nile-blue B	2 i.p. 0.5 s.c. 0.2 s.c. 0.5 s.c.	0	
Phenazines	Janus-green Neutral-red Safranin	0.05 i.p. 1 s.c., also tested 0.1% in diet 0.5 s.c.	0	
Azo-dyes	Trypan-red Trypan-blue Congo-red	0.5 s.c. 0.25 s.c. 1 s.c.	0	Ineffective at 1:4,000 (stronger solution not tested)
Thiazines	Thionine Thiodiphenylamine	2-4 s.c. also tested 0.5% in diet 1% in diet	0	Ineffective at 1:2,000 (no test in rabbit)
Pyronine	Pyronine B	No test	0	1:5,000
Phenazine	Methylene violet 3RA	0.25 s.c.	0	1:25,000
Thiazine	1-Methyl-3-diethylamino-7-(mono) <i>n</i> -butylamino-P*	0.25 s.c.	0	1:4,000
Oxazine	Meldola blue	0.1 s.c.	0	1:50,000
Thiazine	Methylene-blue	1 s.c. (also tested in diet)	+	1:500,000
	Toluidine-blue	1-2 s.c. (also tested in diet)	+	1:500,000
	Methylene-green	1 s.c.	0	1:125,000
"	1-Methyl-3-diethylamino-7-di- <i>n</i> -butylamino-P*	0.25	0	1:500,000
"	1-Methyl-3-diethylamino-7-di- <i>n</i> -propylamino-P*	0.25	0	1:500,000
"	1-Methyl-3 7-bis (diethylamino) P*	0.25	0	1:500,000
"	New-methylene-blue	1 s.c.	+	1:500,000
"	3-Diethylamino-7-di- <i>n</i> -butylamino-P*	1 s.c.	+	1:500,000
Selenazine	Selenium-methylene-blue	2 s.c.	+	1:500,000

\*P=phenazthionium chloride hydrochloride

Table II—mostly subcutaneously Forbisen, which Peterson reported to have some activity, was by our technique weakly active When given by mouth in  $6 \times 25$  mg doses, it reduced the average spot-count from 26 to 5 in one test, and from 47 to 17 in another

*Rabbit Tests*—The last column of Table II shows the final concentration of dye which would inactivate rickettsiae *in vitro* when tested as described earlier It is apparent that a few drugs, those of the last section in the table, are very active in the *in vitro* tests, their effective concentrations being about 250 times less than that of most of the dyes tested All but four of the drugs we examined could by this test be readily classified as either "very active" or "useless"

It will be noticed that three of the thiazines were as active as methylene-blue in the rabbit, but were not active in the mouse test, this is presumably because they were more toxic and could only be given in 0.25 mg doses

### DISCUSSION

All the dyes in Table II, with the exception of the azo-dyes represented by trypan-red, trypan-blue and congo-red are similarly constituted and belong to the class of vital stains They fall into the ortho-quinonoid group of dyes to which Ehrlich attached special significance Presumably they are all taken up to a greater or lesser extent by the rickettsiae, but one half of them, as shown by the *in vitro* test, are comparatively harmless The other half inactivate the rickettsiae for the most part at relatively high dilutions In the mouse test, where one is dealing with a more complex chemotherapeutical system, the dyes have not only to be tolerated by the host but have to enter the cells harbouring the rickettsiae Nevertheless the two tests do show a rough correlation

The results in the absence of further evidence are capable of more than one interpretation, but an acceptable view of the chemotherapeutic process would be to suppose that the vital dyes are adsorbed or absorbed by the rickettsiae in either form of the test and the specific fixation of the dye interferes with some vital function necessary for reproduction of the parasite.

The action of the active thiazines seems unlikely to be necessarily dependent on a photodynamic effect, since dye introduced subcutaneously or *per os* is effective on rickettsiae within the lungs

Comparison of the results of the mouse tests and rabbit tests shows that the latter may have some value as a screening method before resort to the direct test in mice, if allowance is made for toxicity The five compounds effective in mice are amongst those which are most active in the rabbit test. Such correspondence as there is between the two tests must not be taken as necessarily applying to compounds chemically unrelated to these dyes the drugs V147 and V186 and related compounds, which are more effective *in vivo* than any of the dyes tested (Andrewes *et al.*, 1944), fail to show any *in vitro* killing power when mixtures are tested in the rabbit's skin.

## SUMMARY

Dyes have been tested for activity against typhus rickettsiae (a) by holding mixtures of organisms and dye in contact *in vitro* and inoculating into the rabbit's skin to test whether infectivity has been destroyed and (b) by a direct *in vivo* chemotherapeutic test in mice. The results of the two tests, so far as dyes were concerned, were closely parallel. Activity was found only in thiazines and in one selenazine.

We extend our thanks to Mrs Frances Hamilton for her help with some of the inoculations and to Drs L. Hellermann and O. L. Peterson and to Imperial Chemical (Pharmaceuticals), Ltd., for their kindness in supplying us with compounds for test.

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## AMIDINES, GUANIDINES AND ADRENALINE INACTIVATION IN THE LIVER

BY

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In 1897 Langlois showed that injection of suprarenal extract into the mesenteric vein of a dog was less effective in causing a rise of blood pressure than injection of the same quantity of extract into the jugular vein. This difference between the two modes of administration was less when large amounts of the extract were used, and Langlois concluded "Il est facile de supposer que la foie ne peut neutraliser qu'une partie de la substance et que si l'injection est trop forte, un excès passe au début dans la circulation générale". Since the difference persisted even when the injection was made very slowly, it could not be attributed to retardation of the release of the extract into the general circulation. Carnot and Jossierand (1902) demonstrated that adrenaline injected into the portal vein of a dog caused a smaller rise of blood pressure than when injected into the saphenous, femoral or jugular veins. This experiment was confirmed by Elliott (1905), and by many subsequent workers on different species. Bacq (1937) also showed that 0.5  $\mu$ g adrenaline injected into the saphenous vein of the cat under dial anaesthesia caused the same relaxation of the non-pregnant uterus and contraction of the nictitating membrane as 2  $\mu$ g adrenaline injected into the portal vein. Markowitz and Mann (1929) criticized the conclusion that adrenaline was inactivated during its passage through the liver, believing that the smaller pressor effect after injection into the portal circulation was due to its reaching the general circulation in a very much lower concentration. Yet Battelli (1902) showed that the isolated rabbit's liver perfused with defibrinated blood by the portal vein destroyed adrenaline—an observation which was confirmed by Elliott (1905) and by Pak (1926). Philpot and Cantoni (1941) investigated the ratio of equipressor doses of various substances injected into the portal circulation (from a cannula in the splenic vein) and into the jugular vein in the spinal cat. They found the average ratio for adrenaline was 4.7, that is to say, the dose injected into the splenic vein had to be 4.7 times that injected into the jugular in order to obtain the same pressor effect. Since the pressor action of pituitrin was not modified by passage through the liver, they concluded that the criticism offered by Markowitz and Mann was untenable, and that in the liver *in vivo* the main instrument of adrenaline destruction was amine oxidase. They were supported in this conclusion by the observation that methylene blue, which inhibited the destruction of

adrenaline *in vitro* by a liver suspension, greatly augmented the pressor effect of adrenaline injected into the portal vein

A study of the pharmacological action of various amidines (Dawes, 1945), and the observation by Blaschko and Duthie (1944) that pentamidine and propamidine could, like methylene blue, inhibit the destruction of tyramine by a suspension of rabbit liver in a concentration of  $10^{-4}$ , both led to a review of the problem by the method used by Philpot and Cantoni. In this way a number of amidine and guanidine derivatives has been found to increase the pharmacological action of adrenaline and of other sympathomimetic amines injected into the portal circulation. However, not all of these compounds are able to inhibit the action of amine oxidase *in vitro*.

I am indebted to Dr H King, Dr A J Ewins and Mr W A Broom for many of the substances used in these experiments. The V substances and marfanil were obtained through the kindness of Dr J Walker. For the Synthalin B (dodec-methylene diguanidine dihydrochloride) I am indebted to Dr E M Lourie and for the diphenyl guanidine hydrochloride and S-methyl isothiourrea iodide to Dr H R Ing.

Pentamidine (*p p'*-diamidino-1,5-diphenoxypentane) was used throughout as the di-isethionate. The serial number V147 is used in place of the chemical description *p*-sulphonamidobenzamidine hydrochloride.

## METHODS

Most of the experiments were performed in spinal cats prepared by section of the spinal cord at the level of the second cervical vertebra. In order to inject solutions into the portal vein, a cannula was tied either into a small mesenteric vein or into the splenic vein, and the abdomen was closed around the cannula.

## RESULTS

In confirmation of Langlois (1897, 1898) it was observed that the relation between doses of adrenaline producing equal rises of blood pressure by the jugular and by the portal routes differed according to the amount injected. This is shown in Table I, where it is seen that the ratio was 1:5 when the dose injected into the

TABLE I  
DOSES OF ADRENALINE PRODUCING EQUAL RISES OF BLOOD PRESSURE

Intrajugular injection $\mu$ g	Intraportal injection $\mu$ g.	Blood Pressure rise mm Hg
4	20	54
9	40	78
28	80	110
72	160	134

portal vein was  $20\text{ }\mu\text{g}$  adrenaline, and 1.22 when the dose injected was  $160\text{ }\mu\text{g}$ . What does this difference indicate? One explanation is that the liver destroys adrenaline, and that the rise of pressure after intraportal injection depends on the amount of adrenaline escaping into the general circulation through the hepatic veins. If the dose injected into the portal vein is large, a greater proportion will reach the general circulation, and there will be proportionately less difference between the doses injected into the portal and jugular veins which cause the same rise of blood pressure.

### *The Potentiation of Adrenaline Injected into the Portal Circulation*

The pressor action of adrenaline injected into the portal vein of a spinal cat is both increased and prolonged by the simultaneous injection of aromatic

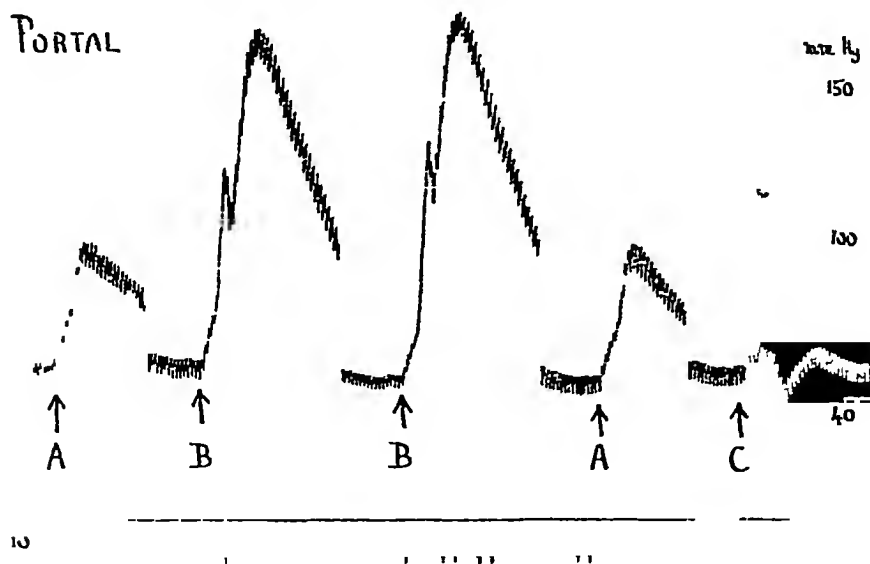


FIG 1—Spinal cat 3.5 kg. Blood pressure record. All injections were made from a cannula in the splenic vein so that the injected fluid entered the portal circulation at 8 minute intervals. The injection of  $40\text{ }\mu\text{g}$ . adrenaline + 1 mg pentamidine isethionate mixed in the same syringe (at B) caused a larger rise of blood pressure than  $40\text{ }\mu\text{g}$ . adrenaline (at A). 1 mg pentamidine isethionate alone (at C) has an insignificant effect.

diamidines and monoamidines, of aliphatic diguanidines, diamidines and monoamidines, and of guanidine itself. Fig 1 shows the potentiation of  $40\text{ }\mu\text{g}$ . adrenaline injected into the portal vein by 1 mg pentamidine. In the same animal 2 mg pentamidine injected into the jugular vein caused a prolonged reduction in the pressor action of  $2\text{ }\mu\text{g}$  adrenaline (Fig 2). There is therefore a striking difference between the action of pentamidine on adrenaline injected by



the portal vein and by the jugular vein, in contrast to cocaine, ephedrine and tyramine, which potentiate the action of adrenaline by both routes. In order to investigate this difference, a closer study was made of the action of amidines on intrajugular and intra-arterial injection.

### *The Action of Amidines on Intrajugular and Intra-arterial Injection*

Both aromatic diamidines (Fig 2 and Wien, 1943) and aromatic mono-amidines (Dawes, 1945) reduce the pressor action of adrenaline when injected into the jugular vein of a spinal cat in large doses. This reduction occurs mainly in the muscle vessels. Fig 3 shows that pentamidine greatly reduces the vasoconstrictor action of adrenaline in the dog's hindlimb, perfused with defibrinated blood from a Dale-Schuster pump. Similarly Wien (1943) produced evidence of the reduction of the vasoconstrictor action of adrenaline by propamidine, in

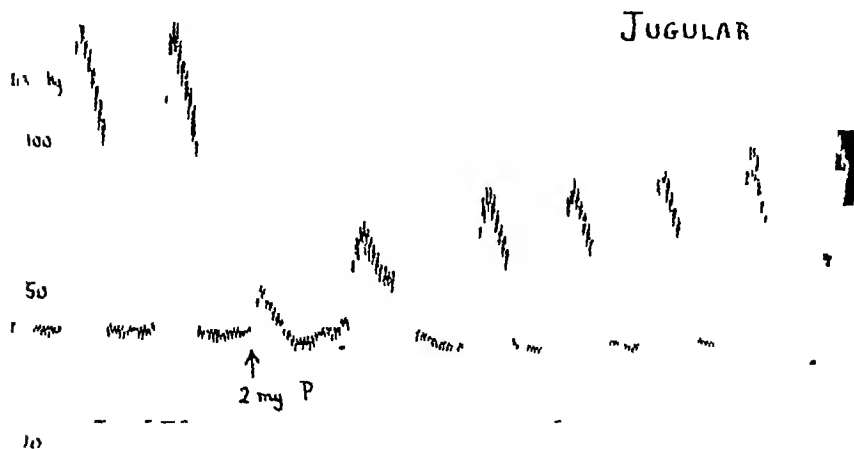


FIG 2.—Same cat as in Fig. 1. All injections made into the jugular vein. The pressor action of 8  $\mu$ g adrenaline (injected at 4 minute intervals) is greatly reduced by 2 mg pentamidine isethionate (P).

the cat's hindlimb perfused with haemoglobin-Ringer solution. In the dog heart-lung preparation, and in the isolated rabbit auricle (suspended in oxygenated Ringer-Locke at 28° C), neither pentamidine nor V147 (*p*-sulphonamido-benzamidine hydrochloride) appreciably modified the action of adrenaline.

These experiments demonstrate that the main site of action of pentamidine in the peripheral circulation is on the vessels rather than on the heart. Now it is well known (Carnot and Josserand, 1902, Livon, 1904, Elliott, 1905) that injection of adrenaline into the femoral artery causes a much smaller rise of blood pressure than intravenous injection. This has been attributed to the slow libera-

tion of adrenaline from the tightly constricted vessels (Markowitz and Mann, 1929), combined with rapid inactivation. The situation bears an obvious resemblance to that following injection of adrenaline into the portal vein. Yet, although pentamidine and V147 greatly potentiate the pressor action of adrenaline injected into the portal vein, they produce no such striking effects on intra-arterial injection. Thus in spinal cats with a cannula in the external iliac artery, so that the injected solution passed into the vessels of the opposite leg, 16–20  $\mu$ g adrenaline were required on intra-arterial injection to cause the same rise of blood pressure as 2  $\mu$ g adrenaline by the jugular vein. In two such

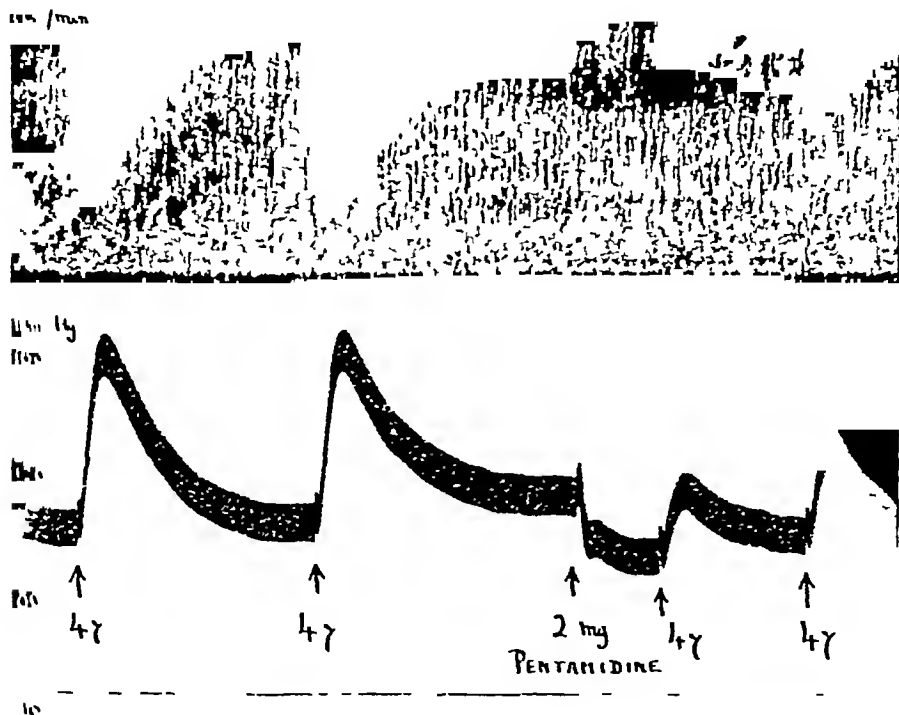


FIG 3—Dog's hindlimb perfused with defibrinated blood. Above: outflow record. Below: pressure in arterial cannula. The injection of 2 mg. pentamidine isethionate greatly reduces the vasoconstrictor action of 4  $\mu$ g. adrenaline.

preparations the simultaneous injection of 0.5–2.0 mg pentamidine or 1–5 mg V147 caused only a tiny increase in the pressor action of adrenaline, whereas the same doses of these compounds more than doubled the response when injected with adrenaline into the portal vein. This difference between intra-arterial and intraportal injection is the more surprising when it is considered that both pentamidine and V147 not only greatly diminish the vasoconstrictor

action of adrenaline in the dog's hindlimb, but themselves have a far greater vasodilator action in the presence of an adrenaline infusion. It would be expected that administration of either of these drugs would allow more adrenaline to escape into the general circulation after intra-arterial injection. The fact that this does not occur leads to the conclusion that there must be some fundamental difference between the reactions of the liver and of the muscle to adrenaline and these amidine derivatives.

#### *The Action of Amidines on Intraportal Injection*

What is happening in the liver when pentamidine or V147 is injected together with adrenaline? The answer to this question must be the key to the difference between intraportal and intrajugular injection. The potentiation of adrenaline on intraportal (but not on intrajugular) injection has been observed

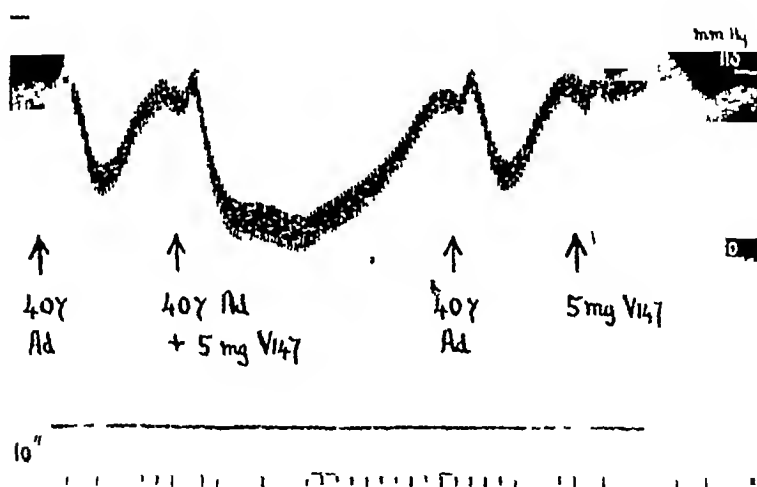


FIG 4—Spinal cat. Blood pressure record. All injections into the portal circulation 3.5 mg. ergotamine tartrate had been injected previously to cause reversal of the pressor action of adrenaline. The injection of 5 mg V147 increases and prolongs the depressor action of 40  $\mu$ g. adrenaline.

in the rabbit under urethane, and in the dog under chloralose anaesthesia, as well as in the spinal cat, and consequently the phenomenon is not confined to one species. Moreover, not only the pressor action of adrenaline, but also the depressor action of small doses (in a cat under ether anaesthesia) is increased by amidines. The actions of adrenaline in causing contraction of the spleen, and of the innervated or denervated nictitating membrane, are also increased. Fig 4 shows the potentiation by V147 of the depressor action of adrenaline injected into the portal vein of a spinal cat, after full ergotamine reversal. These experiments very strongly suggest that less adrenaline is being inactivated during its

passage through the liver in the presence of an amidine derivative, so that more adrenaline reaches the general circulation

In no instance in which adrenaline and either pentamidine or V147 were injected into the portal circulation has the ensuing pressor response been as great as that caused by the injection of an equal quantity of adrenaline alone into the jugular vein of a spinal cat. The potentiation of the pressor action by these substances is therefore due entirely to a reduction in the effect of the liver upon adrenaline. Comparatively small doses of the drugs are required, and some potentiation of adrenaline has been observed with as little as 0.1 mg V147 or pentamidine injected into the portal vein of a spinal cat weighing 2-3 kg. If a short interval is left between the injection of 1-2 mg pentamidine and that of adrenaline, the potentiation is less, and it disappears altogether if the interval is as long as 10 minutes, by this time the greater proportion of the pentamidine absorbed by the liver has been inactivated. There is other evidence that pentamidine itself (as well as adrenaline) is rapidly absorbed from the blood stream during its passage through the liver. For instance 2-4 mg pentamidine injected into the jugular vein of a spinal cat reduced by more than half the pressor action of 10-20  $\mu$ g adrenaline, whereas 2-4 mg pentamidine injected into the portal vein scarcely affected the pressor action of adrenaline injected into the jugular vein.

The reduction in the inactivation of adrenaline during its passage through the liver, caused by the simultaneous administration of amidine derivatives, might be due to inhibition of the enzyme systems responsible for the inactivation of adrenaline, or to interference with the uptake of adrenaline by the liver cells. An alternative explanation might be that these drugs derange the hepatic circulation (by vasodilatation, for instance) and so allow the adrenaline to pass more rapidly through the liver. This last explanation, while it cannot be entirely excluded, is rendered unlikely for a variety of reasons. For instance, if the vasodilatation caused by some of these drugs were the principal cause of the potentiation in the liver, it might be expected that a similar potentiation would be observed after intra-arterial injection into muscle, in which this vasodilatation has been demonstrated (Fig 3) in fact no such potentiation is seen in muscle. Secondly some difference might be expected in the interval between the injection of the solution and, say, the peak response in blood pressure and heart rate. As Fig 5 shows, no difference is observed, whether adrenaline be injected alone or with V147 into the portal vein, in the time characteristics of the initial rise of blood pressure. A close inspection of such tracings also fails to reveal any difference in the time which elapses before the heart rate increases.

Many authors have shown that adrenaline causes a rise in portal pressure after injection into the portal vein of a cat or dog. The subject was extensively reviewed by Bauer, Dale, Poulsson and Richards (1932) and the observations of McMichael (1932) are also relevant. The conclusion that this rise in portal pressure is due to constriction of the portal venous radicles is well substantiated.

Yet, when V147 is injected together with adrenaline from a cannula tied into a small mesenteric vein of a spinal cat, the rise in lateral portal pressure (measured from the splenic vein from a cannula containing Ringer solution and heparin) is, if anything, slightly greater, i.e. V147 does not reduce the constrictor action of adrenaline under these circumstances. Similarly, as Fig 4 shows, V147 still potentiates adrenaline after full ergotamine reversal, when the vasoconstrictor action of adrenaline upon the peripheral circulation has been abolished. The

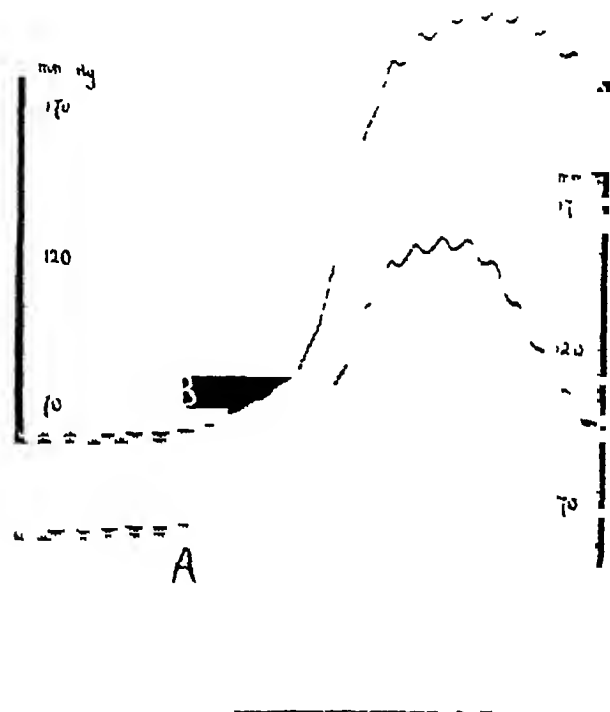


FIG 5—Spinal cat. Blood pressure records of two consecutive observations superimposed. Injections into the portal circulation were accurately timed during the interruption of the upper signal marker. The lower record A was obtained after injection of 20  $\mu$ g adrenaline and the upper record B after 20  $\mu$ g adrenaline + 5 mg. V147.

method of Bauer, Dale, Poulsson and Richards (1932) as modified by Chakravarti and Tripod (1940) was used to perfuse the isolated dog's liver with defibrinated blood. In four such preparations, 4–20 mg V147 injected into the blood entering the portal vein did not appreciably alter the portal pressure, hepatic arterial pressure, liver volume or venous outflow, even during the infusion of adrenaline.

In the same way, various drugs which cause vasodilatation in the peripheral circulation, such as sodium nitrite (2–10 mg) and adenosine (0.2–1.0 mg), do not increase the pressor effect of adrenaline after intraportal injection into spinal cats. Theophylline sodium acetate (10 mg) causes an almost insignificant potentiation. Histamine (2–20  $\mu$ g) is quite inactive. Conversely guanidine and methylguanidine, which cause a rise of blood pressure in the intact animal owing to peripheral vasoconstriction, and which in high concentrations (1–100) cause contraction of isolated arterial strips (Lewis and Koessler, 1927), both potentiate the pressor action of adrenaline after intraportal injection. It therefore seems very improbable that guanidines and amidines reduce the inactivation of adrenaline in the liver simply by causing vasodilatation.

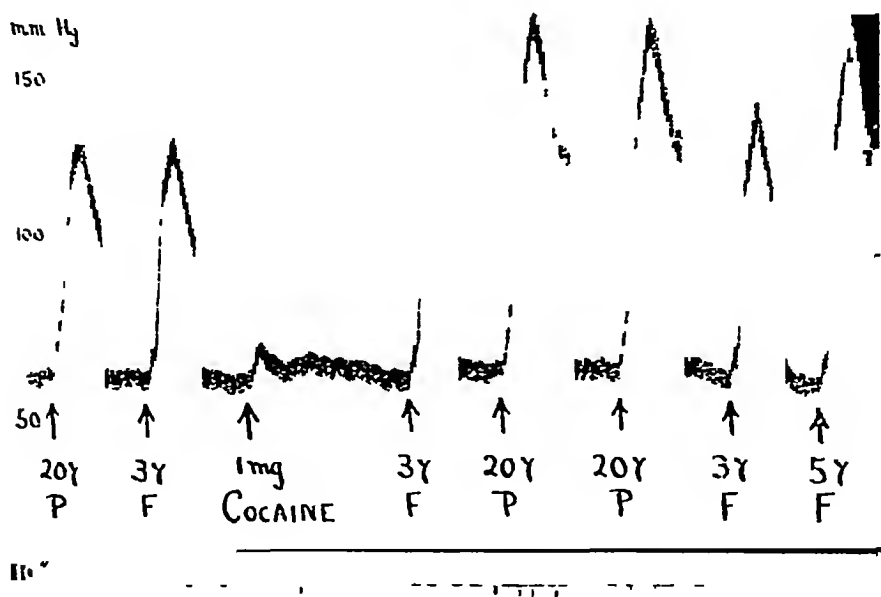


Fig 6—Spinal cat. Blood pressure record. Before injection of cocaine 20  $\mu$ g. adrenaline injected into the portal vein (P) caused the same rise of blood pressure as 3  $\mu$ g. into the femoral vein (F). After 1 mg cocaine hydrochloride injected into the portal vein 20  $\mu$ g. adrenaline intraportally caused the same rise of blood pressure as 5  $\mu$ g. adrenaline intrafemorally.

*Sympathomimetic Amines and Cocaine*—It has already been mentioned that cocaine, ephedrine and tyramine potentiate the pressor action of adrenaline in the spinal cat, after injection into the portal vein. Fig 6 illustrates this for cocaine. 20  $\mu$ g. adrenaline injected into the portal vein caused the same rise of blood pressure as 3  $\mu$ g. injected into the femoral vein, after 1 mg cocaine hydrochloride the pressor action of 20  $\mu$ g. adrenaline intraportally was equal to

that of 5  $\mu$ g intrafemorally (In this experiment the cocaine was injected into the portal circulation to emphasize as far as possible its action on the liver) Ephedrine and tyramine have the same effect, though that of tyramine is more transient.

Amidine derivatives such as pentamidine, V147 and even guanidine itself, on intraportal injection into a spinal cat, increase the pressor action not only of adrenaline, but also (and equally well) that of others of the more active sympathomimetic amines, such as corbasil, epinine and noradrenaline. Less active amines such as *l-m*-sympatol,  $\alpha$ -methyl adrenaline, N-methyl adrenaline and adrenalone, which have to be injected in doses of 0.1–1.0 mg into the portal

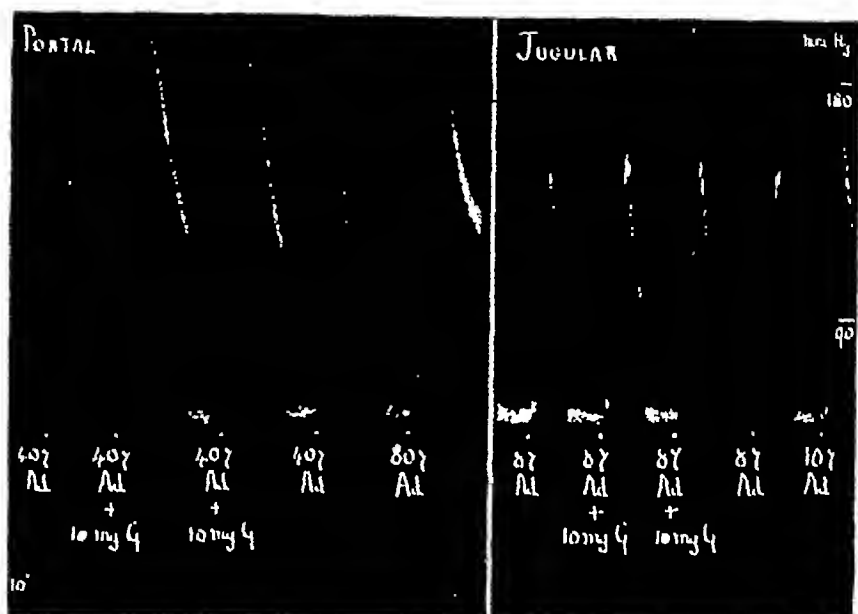


FIG 7—Spinal cat. Blood pressure record 10 mg guanidine hydrochloride greatly increases the pressure action of 40  $\mu$ g adrenaline injected into the portal vein, but scarcely affects the pressor action of 8  $\mu$ g adrenaline injected into the jugular vein

vein to cause a rise of blood pressure, show a much smaller potentiation (relative to that of adrenaline) when the injection is accompanied by an amidine derivative

**Other Amidine Derivatives**—This investigation started with a study of the action of pentamidine and V147 on intraportal injection, but the property of potentiating the action of adrenaline under these circumstances alone is by no means confined to these two drugs. Guanidine hydrochloride itself in a dose of 10 mg will double the response to adrenaline injected into the portal vein, but not via the jugular vein (Fig 7), and the minimal effective dose of guanidine is about 0.5 mg/kg. Both methylguanidine hydrochloride and diphenylguanidine

hydrochloride cause a considerable potentiation of adrenaline on intraportal injection into a spinal cat in a dose of 1 mg, they are slightly more active than guanidine

*Monoamidines of the type*  $\text{CH}_3(\text{CH}_2)_n(\text{NH})\text{NH}_2$

Five representatives of this group were studied, viz. those in which  $n$  was 3 and 4 (sulphates), 8, 10 and 14 (hydrochlorides). The method used for estimating the relative activity of these compounds was to inject adrenaline together with 0.25–0.5 ml of the substance in 0.01M solution into the portal vein, and compare each member of the series with that immediately above and below it. The type of record produced is illustrated in Fig. 8, which shows the relative activity of

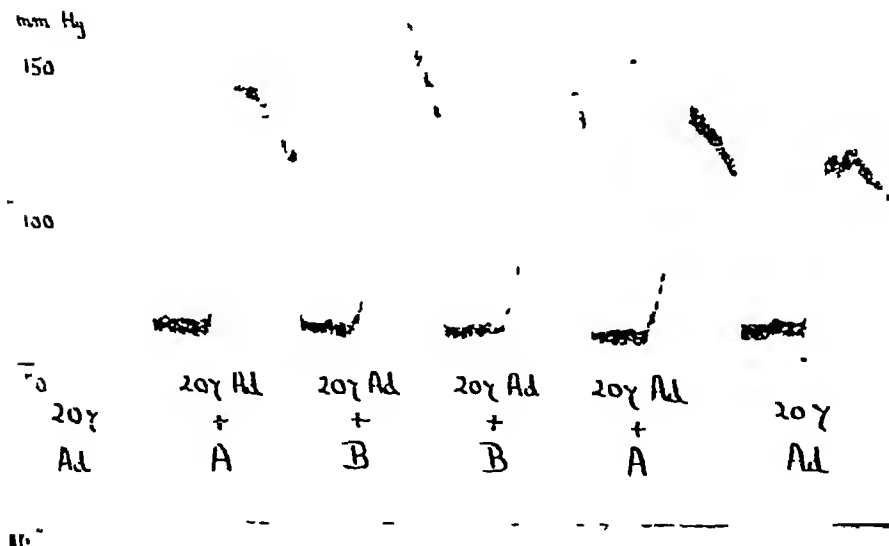


FIG. 8—Spinal cat. All injections into the portal circulation. To show the relative increase of the pressor action of 20  $\mu\text{g}$ . adrenaline by A, 0.25 ml. M/100 lauramidine and by B, 0.25 ml. M/100  $n$ -decane-1 10-diamidine.

$n$ -decane-1 10-diamidine and the corresponding monoamidine (lauramidine) where  $n=10$ . At least two cats were used for each comparison and the point of maximum activity in each series of compounds was confirmed on at least four cats.

In this series of monoamidines, all of which show activity, the strongest was that in which  $n=4$ . That in which  $n=3$  was considerably less active and there was also a progressive decline in activity as the aliphatic chain was lengthened to



8, 10 and 14 The last member of the series ( $n=14$ ) was difficult to dissolve and produced a soapy colloidal solution There is therefore a maximum in this series somewhere about  $n=4-7$ , probably nearer 4 than 7 The intermediate members of the group were unfortunately not available

*Diamidines of the type*  $\text{NH}_2(\text{HN})\text{C}(\text{CH}_2)_n\text{C}(\text{NH})\text{NH}_2$

In this series five compounds were examined, viz those in which  $n=7$  (sulphate) 10, 12, 14 and 16 (hydrochlorides) There was a progressive increase in activity as the chain was lengthened up to  $n=14$ , the compound in which  $n=14$  was more active than that in which  $n=12$  or 16 The most active of the series of monoamidines (that in which  $n=4$ ) was not quite as strong (somewhat more than half as strong) as the most active diamidine (that in which  $n=14$ )

*Diguanidines of the type*  $\text{NH}_2(\text{HN})\text{C.NH}(\text{CH}_2)_n\text{NH.C}(\text{NH}).\text{NH}_2$

Of this series the compounds in which  $n=5, 8, 12, 14$  and 16 (hydrochlorides) were tested There was increasing activity as the chain was lengthened up to  $n=12$  (Synthalin B), the latter was a little stronger than the  $n=8$  or 14 compounds The  $n=16$  compound was difficult to dissolve and yielded a colloidal solution, it was considerably less active. The most active member of this series ( $n=12$ ) was very little stronger than the most active diamidine ( $n=14$ )

*Aromatic Monoamidines*—Three compounds were investigated *p*-amino-benzamidine hydrochloride, V147 (*p*-sulphonamidobenzamidine hydrochloride) and V187 (*p*-sulphomethoxybenzamidine hydrochloride) The principal feature common to these compounds is the benzamidine group  $-\text{C}_6\text{H}_4\text{C}(\text{NH})\text{NH}_2$ , and they all possess considerable activity in reducing the inactivation of adrenaline in the liver In this respect they are only a little less active than the most active diguanidine (Synthalin B where  $n=12$ ) molecule for molecule they are about half as strong

*Aromatic Diamidines*—Four aromatic diamidines were used pentamidine (*p p'*-diamidino-1,5-diphenoxypentane) di-isethionate, propamidine (the corresponding 1,3-diphenoxypropane compound) di-isethionate, *p p'*-diamidino-1,2-diphenoxyethane dihydrochloride and stilbamidine (*p p'*-diamidinostilbene) di-isethionate All four were about twice as active as the aromatic monoamidines, and therefore of the same order of activity as Synthalin B Molecule for molecule pentamidine and Synthalin B are approximately 50 times as active as guanidine

*The Effect of Changes in the Amidine Group*—The amidine group  $-\text{C}(\text{NH})\text{NH}_2$  was found to be highly specific in causing a reduction in the inactivation of adrenaline by the liver A number of modifications of the group were investigated, all of which greatly decreased the activity of the parent compound Thus marfanil (*p*-sulphonamidobenzylamine hydrochloride) and V335 (*p*-sulphomethoxybenzylamine hydrochloride) are the benzylamines ( $-\text{C}_6\text{H}_4\text{CH}_2\text{NH}_2$ ) corresponding to the two benzamidines V147 and V187,

the benzylamines have only 1/20 of the activity of the benzamides V186 (*p*-sulphonamidobenzamidoxime hydrochloride) is the benzamidoxime ( $-C_6H_4C(NO)NH_2$ ) corresponding to V147, and has 1/100 of the activity of the latter. *p*-Aminobenzoic acid and *p*-aminobenzene sulphonamide (the aniline ( $-C_6H_4NH_2$ ) compound corresponding to V147) also have only 1/20 of the activity of *p*-aminobenzamide hydrochloride. Similarly semicarbazide hydrochloride ( $NH_2CO.NH.NH_2$ ) was much less active than guanidine hydrochloride. Phenylbenzamide hydrochloride ( $C_6H_5C(NH)NHC_6H_5$ ) was also considerably less active than *p*-aminobenzamide hydrochloride. It is evident that among the various substances which were tested, those containing the amidine group were the most active.

*Composition of the Rest of the Molecule*—In compounds containing an amidine group small alterations in the other parts of the molecule influence the activity very greatly. This has already been demonstrated for aliphatic mono-amidines, diamidines and diguanidines. The introduction of a carboxyl group, as in creatine and arginine, reduces the activity considerably. Creatine in doses of 15 mg. in a spinal cat occasionally caused a slight potentiation, 10 mg. arginine was virtually inactive, but 50 mg. caused a slight potentiation. Both were less active than guanidine, molecule for molecule.

Table II gives a list of other substances which also were relatively inactive, and of the doses in which they were injected into spinal cats.

TABLE II

<i>Inactive Substances</i>	<i>Dose</i>
Urea	20-30 mg.
Thiourea	80 mg.
Thiouracil (relatively insoluble)	1 mg.
S-methyl isothiouraea iodide	1 mg.
Alloxan	10-30 mg.
Sulphaguanidine	2 mg.
Dodecamethylene di-isothiouraea dihydrochloride	1 mg.

## DISCUSSION

There is a tendency in the literature to over-emphasize the importance of the liver in the physiological inactivation of adrenaline yet as long ago as 1905 Elliott concluded "that adrenaline disappears in the tissues which it excites". Markowitz and Mann (1929) showed that exclusion of the liver in dogs caused only a slight increase and prolongation of the rise of blood pressure produced by injection of adrenaline, and they suggested that though the liver may play some part in the destruction of adrenaline, this part is no greater than can be accounted for by as much of the vascular tree as is contained therein. Bacq (1937) came to the same conclusion, using the nictitating membrane of the cat as an index of adrenaline activity after temporary occlusion of the circulation to the intestines.

or after total evisceration. This view receives further support from the experiments with guanidine and amidine derivatives. If the liver played any great part in the inactivation of adrenaline in the general circulation, the potentiation by amidines of adrenaline injected into the portal circulation would be matched by a similar potentiation on intrajugular injection, whereas in fact the latter is so small that it was not discovered until particular search was made. The principal physiological problem must therefore be the inactivation of small quantities of adrenaline outside the liver, and the work which has been done on adrenaline inactivation in the liver can only be applied elsewhere by analogy.

**Mono-amine Oxidase**—Ephedrine (Blaschko, Richter and Schlossman, 1937), cocaine (Philpot, 1940), and methylene blue are known to inhibit mono-amine oxidase *in vitro*. Philpot and Cantoni (1941) have shown that the pressor effect of adrenaline injected into the portal circulation of spinal cats was greatly augmented by methylene blue, and in this paper the same was found to be true of ephedrine and cocaine. Yet it seems very improbable that amidine and guanidine derivatives reduce the inactivation of adrenaline in the liver by their action on mono-amine oxidase. For the aromatic mono-amidine V147 and guanidine scarcely inhibit mono-amine oxidase at all (Blaschko and Duthie (1945), and personal communication), and pentamidine (which inhibits mono-amine oxidase strongly) potentiates the pressor action of corbasil on intraportal injection, in spite of the fact that corbasil, because of the  $-\text{CH}_3$  group on the  $\alpha$ -carbon atom of its side chain, is not attacked by mono-amine oxidase. There are two possibilities. First, mono-amine oxidase may play an insignificant part in the inactivation of adrenaline in the liver, if this is so we have to seek another explanation for the potentiation of adrenaline by tyramine, ephedrine, cocaine and methylene blue on intraportal injection. It is, for instance, possible that they inhibit the conjugation of adrenaline in the liver to form a sulphuric acid ester (Cf Richter, 1940). Secondly, mono-amine oxidase may play the main part, but amidine derivatives may prevent adrenaline ever reaching it, by interfering with the uptake of adrenaline by the liver cells.

**Bactericidal and Toxic Actions of Amidines and Guanidines**—Other possibilities may be considered. As the experiments with pentamidine show, it seems very likely that amidines and guanidines are rapidly absorbed and metabolized by the liver. It is improbable that they reduce the inactivation of adrenaline by dilating the liver vessels, and so reducing the time available for adrenaline to diffuse into the liver cells. An attempt to show that the reticulo-endothelial system was responsible for the uptake of adrenaline also failed, blocking it with Thorotrast (Maher, 1944) or indian ink did not increase the pressor action of adrenaline injected into the portal vein of a spinal cat.

In 1938 King, Lourie and Yorke examined some of the diamidine and diguanidine derivatives used in this investigation for trypanocidal activity and toxicity. Fuller (1942) studied their action upon a wide range of cocci, anaerobes, dysentery and other Gram-negative organisms in broth and serum. More recently

Blaschko and Duthie (1945) have investigated their action upon mono-amine oxidase *in vitro*. Their toxicity and hypoglycaemic action have also been studied by Bischoff, Sahyun and Long (1929) and by Broom (1936). The activity of these series of drugs increased with the length of the carbon chain up to a maximum, and decreased as the chain was lengthened still further, whether they were tested for toxicity, hypoglycaemic, trypanocidal or antibacterial action, as inhibitors of mono-amine oxidase or for their effect in potentiating the pressor action of adrenaline injected into the portal vein of spinal cats. The chain lengths at which maximum activities were observed, using these four types of measurement, are recorded in Table III. King, Lourie and Yorke (1938) and Fuller (1942)

TABLE III

Series	Chain Length (n) for Maximal Activity					
	King <i>et al</i> (1938) Trypano- cidal	Fuller (1942) Bacterio- static	Blaschko & Duthie Inhibition of mono- amine oxidase	Hypo- glycaemia	Toxicity mice	Potentia- tion of adrenaline on intra- portal injection
$\text{CH}_3(\text{CH}_2)_n\text{C}(\text{NH})\text{NH}_2$ $\text{NH}_2(\text{HN})\text{C}(\text{CH}_2)_n$ $\quad \quad \quad \text{C}(\text{NH})\text{NH}_2$	11	12-15	10 12	3 (Broom) 8 (Broom)	4 (Broom) 10 (Broom) 10-18 (King)	4 14
$\text{NH}_2(\text{HN})\text{C}(\text{NH})(\text{CH}_2)_n$ $\quad \quad \quad \text{NH C}(\text{NH})\text{NH}_2$	10-14	12-18	14	10 (Bisch- off <i>et al</i> )	12-16 (King)	12

examined only two aliphatic mono-amidines (those in which  $n=14$  and 16) and these had little or no activity. The discrepancy between Blaschko and Duthie's figure for the chain length for maximal activity in the mono-amidine series ( $n=10$ ), and that obtained from intraportal injection into spinal cats ( $n=4$ ), provides yet another illustration of the lack of correlation between the inhibition of mono-amine oxidase *in vitro* and the potentiation of adrenaline *in vivo*. With the diamidines and diguanidines, however, the agreement as to the chain length for maximal activity is better. In the diamidine series this is covered by the range  $n=8-18$ , and in the diguanidine series by  $n=10-18$ . Fuller (1942) remarked that the increase of activity with chain length in his series suggested that surface active properties might be in part responsible for the activity of these compounds, and that they might act by combining with and denaturing some essential protein constituent. This would provide an explanation for the agreement in the chain length for maximal activity in such diverse biological measurements. But whereas Fuller (1942) found that for bacteriostatic activity the difference caused by interchange of the end groups (with isothiourreas, amines, amidines, guanidines and quaternary ammonium salts) was not large, in the present investigation the specificity of the amidine (or guanidine) group was remarkably high.

The pharmacological evidence is not in agreement with Fuller's further suggestion that these drugs may be general protoplasmic poisons. For instance, while V147 in a dose of as little as 0.1 mg will potentiate the pressor action of adrenaline after intraportal injection into a spinal cat, it is singularly free from any toxic effect upon the heart (Dawes, 1945). On the other hand, there is ample evidence that guanidine derivatives in general cause liver damage in lethal doses, a point which accounts for the close correlation (in homologous series) in the chain length for maximal toxicity and for maximal hypoglycaemic action (Table III). This evidence requires no amplification for guanidine or Synthalin, and the work of Bischoff *et al* (1929) and of Broom (1936) suggests that many other guanidines, mono-amidines, alkylene diamidines and aromatic mono-amidines also cause liver damage in experimental animals. More recently Daubney and Hudson (1941), Wien, Freeman and Scotcher (1943) and Allen, Burgess and Cameron (1944) have made similar observations with aromatic diamidines in animals, and there has unfortunately been confirmation of their conclusions in man (e.g. Kirk and Henry, 1944). Hawking and Smiles (1941) injected stilbamidine subcutaneously into mice and observed the viscera under ultra-violet light. The fluorescence of stilbamidine gave a rough idea of its distribution, and "judging by the appearances it would seem that the diamidinostilbene collects especially in the liver and kidney". There is therefore evidence for a more general hypothesis. It has not proved possible from the available data to decide upon the specific means by which amidines and guanidines reduce the inactivation of adrenaline during its passage through the liver, but it is possible that this reduction is one easily measurable manifestation of the toxic action of these drugs upon the liver. Indeed one might suggest that all new drugs containing the amidine group which are likely to be used clinically should be examined to see whether they increase the pressor action of adrenaline on injection into the portal vein of a spinal cat. This test is far more rapidly performed than conventional liver function tests, which, of course, it could never replace. But if a new drug did possess a high degree of activity in reducing the inactivation of adrenaline in the liver, then the greatest caution would be required in assessing its action on liver function, and in its preliminary trials on man.

#### SUMMARY

1 Adrenaline injected into the portal vein of a cat, rabbit or dog has less action on the circulation, spleen or nictitating membrane than when injected into the jugular vein. This difference between intraportal and intrajugular injection is decreased by the simultaneous administration of aromatic and aliphatic diamidine and mono-amidine derivatives, of diguanidines and guanidine itself. These substances are therefore believed to reduce the inactivation of adrenaline during its passage through the liver.

2 The inactivation of adrenaline during its passage through the liver is also reduced by ephedrine, tyramine and cocaine. But whereas the latter also

potentiate the action of adrenaline in the peripheral circulation, amidine and guanidine derivatives either decrease it or have no effect.

3 The amidine group  $-C(NH)NH_2$  was found to be highly specific in producing a reduction of adrenaline inactivation in the liver. Closely related benzylamine, aniline and benzamidoxime derivatives did not possess this property in a comparable degree.

4 The basis for the reduction of the inactivation of adrenaline in the liver by amidine and guanidine derivatives is not known. It is not due to inhibition of mono-amine oxidase, but the facts could be explained by these drugs interfering with the penetration of adrenaline into the liver cells.

5 The variation in activity with change in chemical structure in a series of amidine and guanidine derivatives was examined. In two homologous series of aliphatic diamidines and diguanidines the chain length for maximal activity in reducing adrenaline inactivation in the liver was found to be very similar to that recorded by other observers for maximal toxicity, hypoglycaemic, trypanocidal and bactericidal activity, and activity in inhibiting mono-amine oxidase *in vitro*.

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# OBSERVATIONS ON THE ISOLATED PHRENIC NERVE DIAPHRAGM PREPARATION OF THE RAT

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The work which led up to the investigation described in this paper was concerned with the influence of adrenaline on the action of acetylcholine or on tissue functions normally elicited by acetylcholine. Previously experiments were carried out on animals with a normal circulation or on perfused organs, and any adrenaline effects observed were accompanied by vasoconstriction, so that it was conceivable that its action was due to vascular changes.

The improvement in the transmission of impulses along the motor nerve (Bulbring and Burn, 1939) or the lowering of threshold to submaximal stimuli applied to the sciatic nerve (Bulbring and Whitteridge, 1941) by injecting adrenaline into the circulation might have been due to changes in the distribution of blood in the vascular bed of the nerve trunk leading to an alteration of electrical resistance. However, there was a discrepancy in time relations, the increased nervous excitability lagging behind and long outlasting the vascular effect of adrenaline.

In fatigued skeletal muscle adrenaline—and other vasoconstrictor substances—augment muscle contractions (Bulbring and Burn, 1940), but Maibach (1928) and Corkill and Tiegs (1933) obtained the same result on frog muscle suspended in a bath, showing thereby that the action of adrenaline on the fatigued nerve muscle preparation can be observed without its vascular action. Presumably, in fatigue, neuromuscular transmission gradually fails and is restored by adrenaline. When, for instance, Bulbring and Whitteridge (1941) recorded muscle tension and nerve action potentials, a striking absence of parallelism between the effect of adrenaline on muscle and nerve was observed. At a time when no effect of adrenaline on the nerve response to maximal shocks at 1 per sec could be seen, 100 per cent increase was seen in the fatigued muscle.

In non-fatigued skeletal muscle stimulated indirectly with maximal shocks, it was found that adrenaline augmented the effect of prostigmine. The results were taken to indicate that adrenaline facilitates neuromuscular transmission and increases the action of acetylcholine on the muscle (Bulbring and Burn, 1942). Though the dose of adrenaline used was always the same, producing a similar degree of vasoconstriction each time, its effect on the muscle was dependent not only on the presence of prostigmine and on the amount of prostigmine given, but

also and chiefly on the rate of stimulation, i.e. on the rate of accumulation of acetylcholine produced at the motor ending. Nevertheless, vascular changes along the nerve might have occurred and it seemed desirable to repeat the experiments on an isolated mammalian nerve-muscle preparation in which the vascular action of adrenaline is without effect.

Dale and Gaddum (1930) described experiments on the denervated kitten's diaphragm suspended in a bath. Therefore an attempt was made to dissect a slip of kitten's diaphragm with the phrenic nerve attached, to suspend it in an isolated organ bath and record the contractions to nerve stimulation. But even with single shocks at a slow rate (5 per min) the muscle twitches slowly declined, the oxygenation presumably being insufficient. The thinner muscle of a rat's diaphragm was, however, found to work satisfactorily and was used for all the experiments to be described.

### METHOD

Adult rats were used. The rat was killed and bled out. After removing the skin over the chest, the thorax was opened along the right side of the sternum and the frontal part of the right thoracic wall was removed. The mediastinum behind the sternum was severed

and a cut was made just above the frontal insertion of the diaphragm taking care not to damage the phrenic nerve, which sometimes attaches itself to the ribs. The frontal part of the left thoracic wall was then removed and the phrenic nerve was seen quite distinctly. Both left lobes of the lung were removed. The left abdominal muscles were cut along the costal margin and the last rib was held with a pair of forceps. A strip of diaphragm was now cut out. Two converging cuts were made through the ribs towards the tendinous part of the diaphragm parallel to its muscle fibres, 3 mm. to the right and 3 mm. to the left of the point where the phrenic nerve enters the diaphragm. The strip was cut out beyond the tendinous part with about 2.5 cm. of phrenic nerve attached to it. The preparation had a fan-like shape, being 3 mm. wide at the tendinous end, while at the costal margin it was about 12 mm. wide. The preparation was fixed by a glass rod with a pin pushed through an intercostal space holding it at the bottom of the organ bath. A thread tied around the tendinous end was attached to a light isotonic heart lever writing on a smoked drum. The nerve was stimulated by a pair of electrodes of the following design (see Fig. 1). Two platinum plates were enclosed in a small block of perspex with wires running up in a perspex handle 6 cm. long. The platinum plates were 1 mm. thick and 3 mm. square, they were lying parallel in the perspex block, 5 mm. square and 6 mm. thick, in such a way that they were separated by 2 mm. perspex. A hole about 1 mm. wide was drilled centrally through this block containing the plates to take the phrenic nerve which thus passed through a tube of perspex with two platinum rings. This arrangement ensured a good contact for the electrical stimulation and also provided a moist chamber for the nerve, preventing it from drying up. The electrodes were fixed vertically just touching

FIG 1—Perspex electrodes

the surface of the solution inside the bath. After the phrenic nerve had been pulled up through the hole a moist piece of cotton wool was placed on top. Single shocks were applied



to the nerve from a neon lamp circuit at rates varying from 5 to 50 per min. Constant muscle contractions could be obtained for many hours in response to single shocks at rates not exceeding 12 per min. With faster rates fatigue set in if they were applied for prolonged periods. A tetanus was not maintained by this isolated preparation.

Some experiments were performed using fluid electrodes (Collison, 1933) in which the nerve was stimulated in a chamber sealed off with wax at the bottom so that the fluid surrounding the nerve at the point of stimulation was not in contact with the fluid surrounding the muscle.

Tyrod's solution containing double the usual amount of dextrose was used. A sintered glass gas-distribution-tube was fixed at the bottom of the bath providing vigorous oxygenation with a mixture of 95 per cent  $O_2$  + 5 per cent  $CO_2$ . The capacity of the bath was 100 c.c. and the temperature was kept between  $36^\circ$  and  $37^\circ C$ .

## RESULTS

### 1 The Action of Adrenaline

When the phrenic nerve was stimulated with single *maximal* shocks a series of muscle contractions of equal height was obtained. The tension developed in different preparations varied from 10–25 g. It remained constant for several

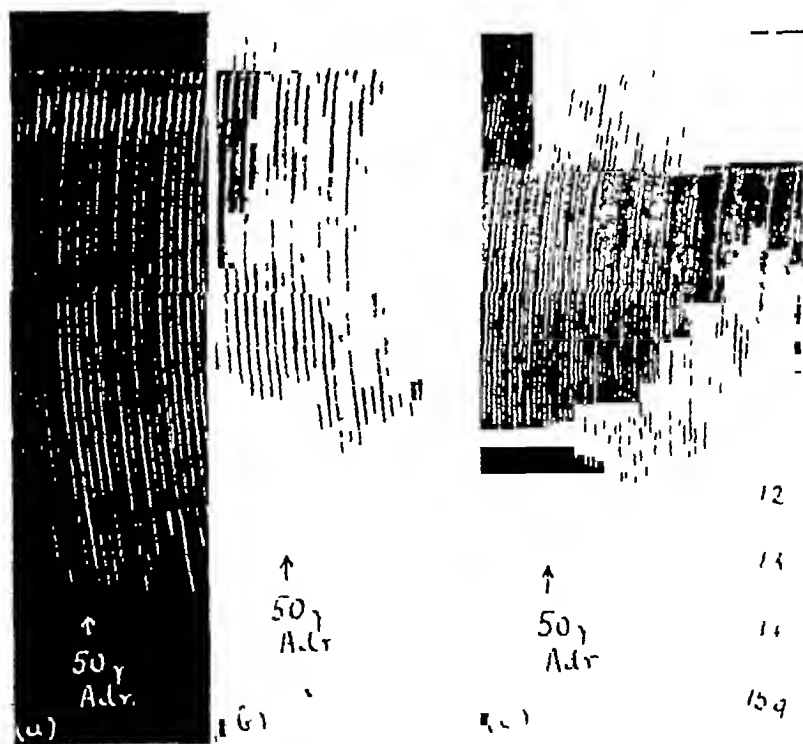


FIG 2—Contractions of rat's diaphragm. (a) and (b) stimulation of phrenic nerve with open electrodes, (c) with fluid electrodes. The effect of  $50 \mu g$  adrenaline on the muscle response to maximal (a) and submaximal single shocks (b) and (c).

hours if the rate of stimulation did not exceed 12 per min. With faster rates the muscle slowly became fatigued. While the muscle contracted maximally and showed no sign of fatigue, the addition of adrenaline to the bath had no effect on the size of contractions. However, when the phrenic nerve was stimulated with *submaximal* shocks, the muscle contractions were slightly increased by the addition of 10  $\mu\text{g}$ –50  $\mu\text{g}$  adrenaline to the bath. This increase never exceeded 20 per cent. In Fig. 2a is shown a record of maximal contractions of the diaphragm, their size was not affected by adrenaline. In Fig. 2b the same muscle was stimulated with submaximal shocks, 50  $\mu\text{g}$  adrenaline now caused a bigger muscle response. When this experiment was repeated on another preparation using fluid electrodes (Collison, 1933) the same result was obtained (Fig. 2c). The chamber in which the nerve was enclosed for stimulation was sealed off with wax at the bottom. Thus the adrenaline which was added to the bath had no direct access to the nerve at the site of stimulation and therefore presumably exerted its action nearer to the muscle. In Fig. 3 is shown another experiment in which fluid electrodes were used. The action of adrenaline on maximal stimuli at a slow rate of 6 per min (a), was compared with that on submaximal stimuli at the same rate (b). A similar adrenaline effect was observed on submaximal stimuli at a faster rate of 18 per min (c). Maximal stimuli were then applied (d) and after prolonged stimulation fatigue reduced the muscle contractions to the same size as they had been initially in response to the submaximal stimulation in (c). The adrenaline effect was very similar both in (c) and (d), suggesting an improvement of transmission in both, which was maintained in (c) but not in the fatigued muscle (d).

## 2 The Action of Prostigmine and Eserine

As adrenaline had no effect by itself upon maximal muscle twitches the modification of the action of prostigmine and eserine by additional adrenaline was studied with maximal stimuli only. The effect of prostigmine was found to be dependent on the dose and on the rate of stimulation. With slow stimulation

TABLE I

THE EFFECT OF PROSTIGMINE ON THE SIZE OF MUSCLE CONTRACTIONS ELICITED BY MAXIMAL SINGLE SHOCKS

Dose of Prostigmine	Rate of stimulation per min			
	5–8	9–12	14–16	18–24
0.1 $\mu\text{g}$ –0.2 $\mu\text{g}$	no effect or increase	increase	increase	increase
0.25 $\mu\text{g}$ –0.3 $\mu\text{g}$	increase	increase	uncertain	—
0.4 $\mu\text{g}$ –0.5 $\mu\text{g}$	increase	uncertain	increase followed by depression	—
1–2 $\mu\text{g}$	uncertain	increase followed by depression	depression	depression
10 $\mu\text{g}$	depression	—	—	—

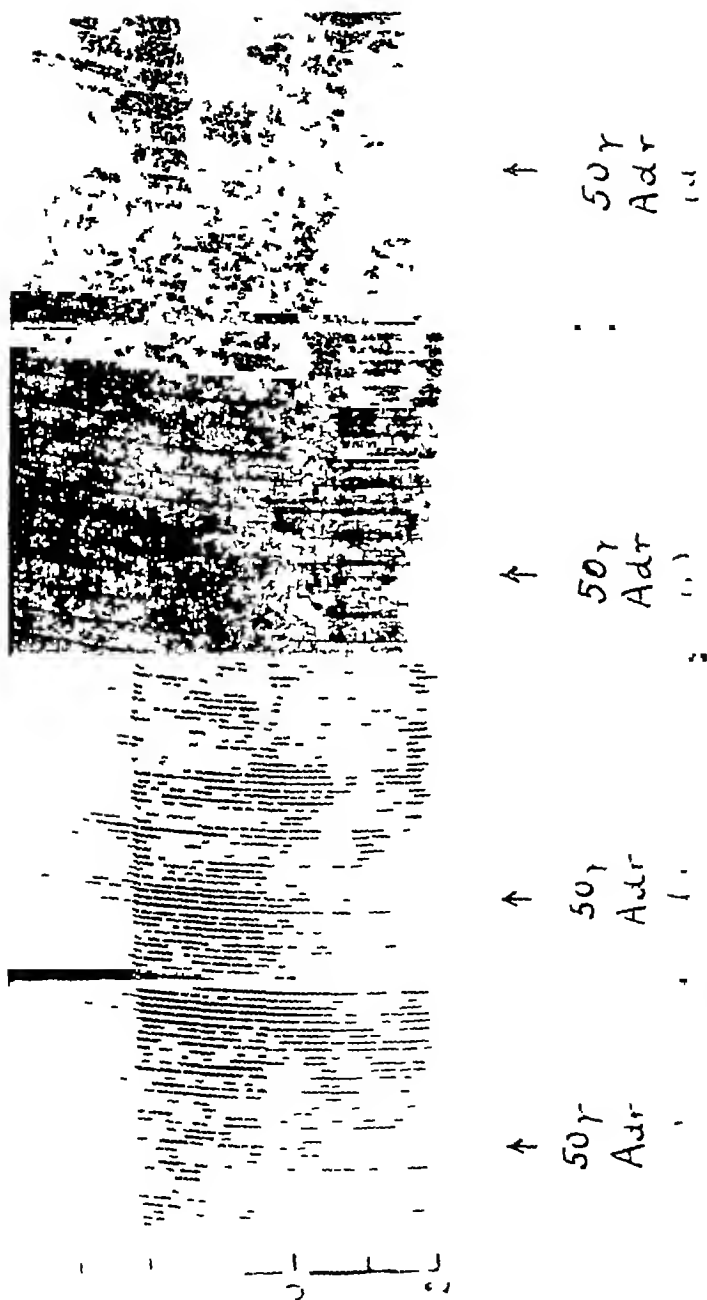


FIG. 3.—Rat's phrenic nerve-diaphragm preparation. Comparison of the adrenaline effect at different rates of stimulation. (a) maximal, 6 per min, (b) submaximal, 6 per min, (c) submaximal 18 per min, (d) muscle fatigued by maximal stimulation 18 per min.

(5 per min) the threshold dose producing increased tension was about  $0.1 \mu\text{g}$  prostigmine or a concentration of 1 in 1,000 million. The accompanying table was compiled from observations in 20 different experiments.

It can be seen from Table I that small doses of  $0.1 \mu\text{g}$ – $0.2 \mu\text{g}$  prostigmine caused an increase of muscle contractions at slow and at fast stimulation rates up to 24 per min. The effect of ten times that amount of prostigmine was uncertain, sometimes producing a temporary augmentation followed by depression.

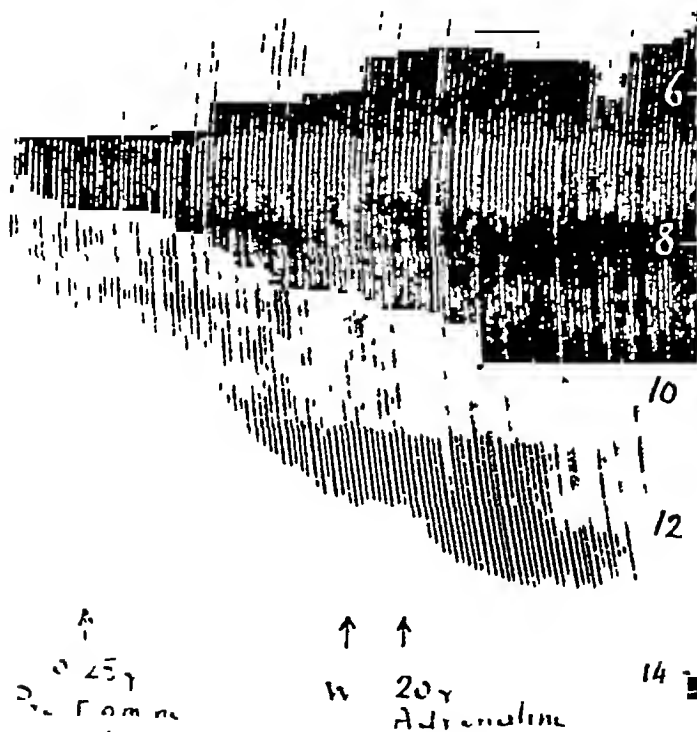


FIG 4—Rat's phrenic nerve-diaphragm preparation. Maximal single shocks 9 per min. Prostigmine causes an increase of muscle contractions, and after a wash out (W) adrenaline causes a further augmentation.

At a slow rate of stimulation, as is seen from Table I, a small dose of prostigmine might either have no effect or cause a rise in muscle contractions. If now adrenaline was added to the bath a further augmentation was sometimes observed. But mostly the adrenaline had no apparent effect unless the prostigmine was washed out before the adrenaline was added. After doses of prostigmine exceeding  $0.3 \mu\text{g}$  or with faster stimulation, adrenaline usually caused depression of the muscle contractions, but again an augmentation was almost always seen if the prostigmine was first washed out.

Fig 4 shows the contractions of the diaphragm in response to maximal shocks at 9 per min. A dose of  $0.25 \mu\text{g}$  prostigmine added to the bath increased the size of contractions. After washing out,  $20 \mu\text{g}$  adrenaline caused a further increase. If the prostigmine was not washed out, the opposite effect was often observed. In Fig 5 the rate of stimulation was faster, 18 per min. After  $0.1 \mu\text{g}$  prostigmine, which by itself scarcely affected the size of contractions, had been washed out,  $10 \mu\text{g}$  adrenaline caused an augmentation (Fig 5a), but when the same small dose of  $0.1 \mu\text{g}$  prostigmine had been repeated, again producing no

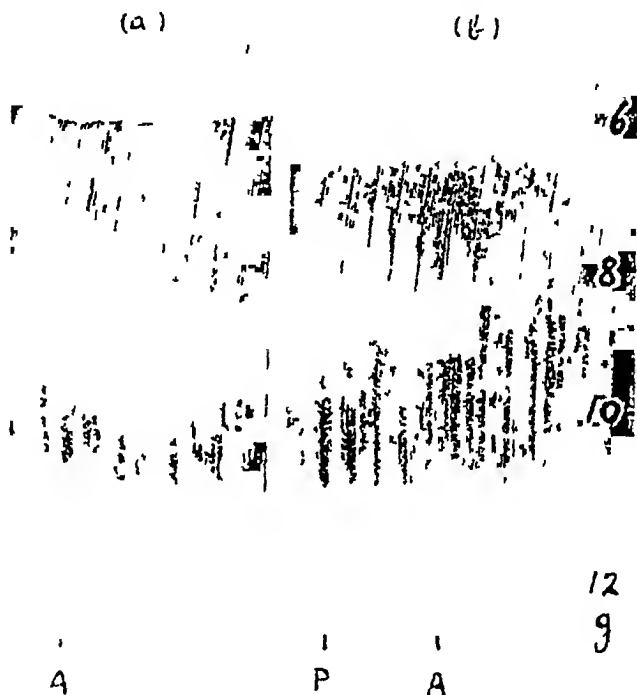


FIG 5—Rat's phrenic nerve-diaphragm preparation. Maximal single shocks 18 per min.  $0.1 \mu\text{g}$  prostigmine had been given beforehand and was washed out, in (a)  $10 \mu\text{g}$  adrenaline (A) caused a rise in muscle contractions. In (b) when  $0.1 \mu\text{g}$  prostigmine (P) was not washed out  $10 \mu\text{g}$  adrenaline (A) caused a fall in muscle contractions.

immediate effect of its own, and was followed 2 min later by  $10 \mu\text{g}$  adrenaline, a depression resulted as shown in Fig 5b.

It may be assumed that the isolated muscle, without a circulation to remove added prostigmine, is progressively affected by the drug, however small the dose, as long as it is left in the bath. Thus more and more acetylcholine is prevented from destruction by cholinesterase. If adrenaline is now added as well, it can only increase muscle contractions before the stage of acetylcholine excess is

reached. The augmentation should therefore be shown best after the prostigmine has been removed from the bath. This is demonstrated in the experiment shown in Fig 6. The rate of stimulation was 7 per min, 0.1  $\mu$ g prostigmine was added to the bath. It increased muscle contractions and was allowed to act for 3 min, when it was washed out. Two minutes later adrenaline was added causing a further increase. After the washing out, the muscle contractions slowly returned to their initial level. The dose of 0.1  $\mu$ g prostigmine was now repeated and allowed to act for 5 min causing an augmentation which was, however, less than that produced by the addition of adrenaline before. When now adrenaline was

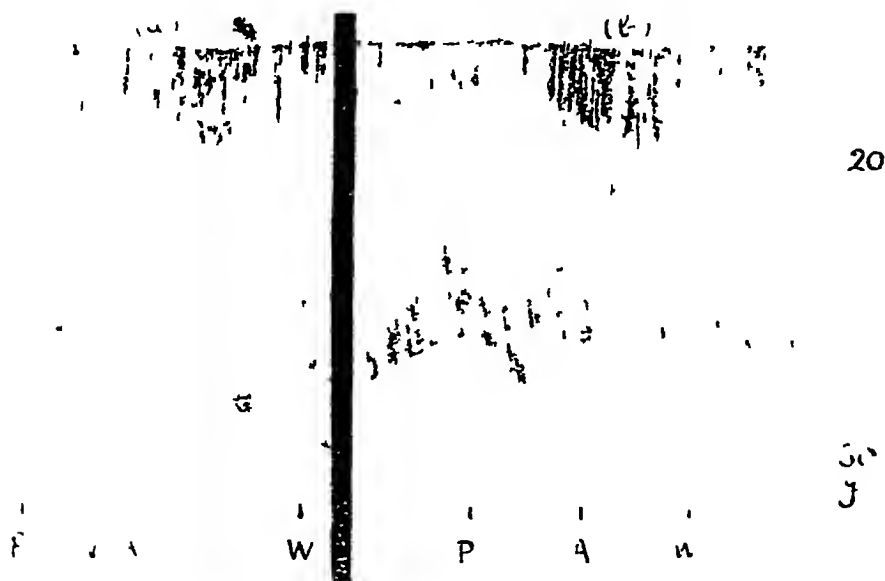


FIG 6—Rats phrenic nerve-diaphragm preparation. Effect of adrenaline (a) after and (b) before washing out a previous dose of prostigmine. Maximal single shocks 7 per min. P=0.1  $\mu$ g. prostigmine, W=wash, A=10  $\mu$ g. adrenaline.

added no effect was seen, the size of muscle twitches slowly declining until the solution was changed. The muscle contractions, instead of returning to their initial size, now gradually increased as though the full adrenaline effect only developed at this stage. Similar observations were made repeatedly and an example is shown in Fig 7. The rate of stimulation was 14 per min in this experiment. A dose of 0.1  $\mu$ g prostigmine produced three times an almost identical augmentation (P). The first dose was washed out, whereupon adrenaline caused its typical further augmentation ( $A_1$ ). After the solution had been changed, the muscle contractions returned to normal. The second dose of prostigmine was not washed out, whereupon adrenaline ( $A_2$ ) caused a slight depression. After another wash out, the muscle contractions increased and a



further rise was obtained with adrenaline ( $A_3$ ). When this was washed out, contractions once more returned to normal. The third dose of prostigmine was washed out but no adrenaline was added until 10 min later, when it produced its typical rise ( $A_4$ ).

There is no qualitative difference between the action of prostigmine and that of eserine, but they differ quantitatively. The threshold dose of eserine was found to be as low as  $0.01 \mu\text{g}$  or 1 in 10,000 million, which produced a rise in muscle contractions during slow stimulation at 5 per min. In a fresh preparation

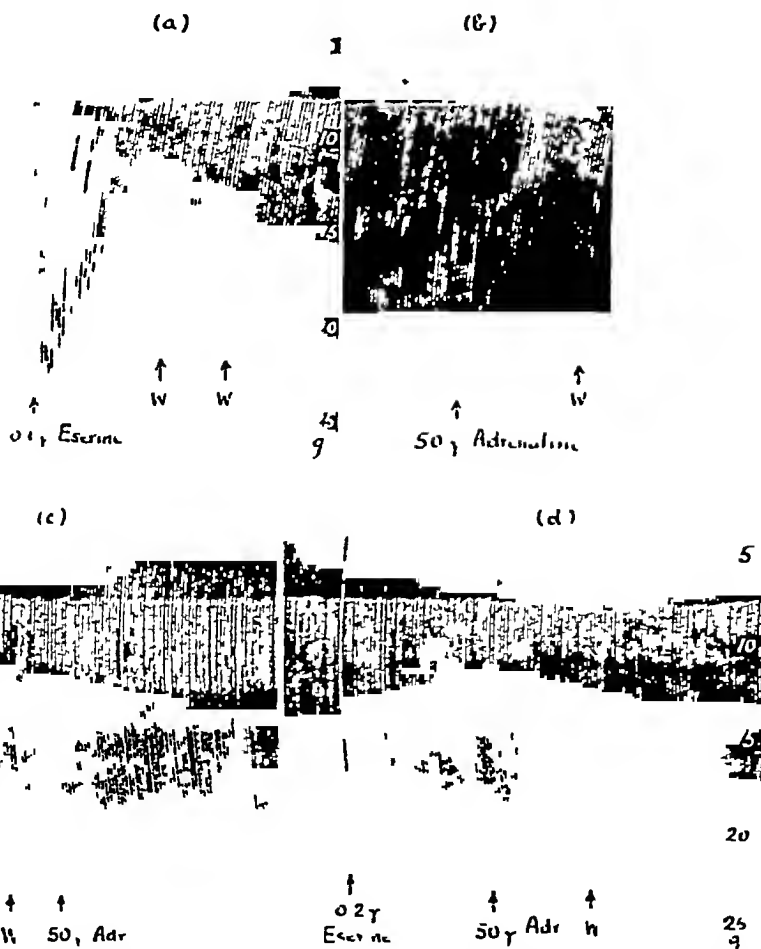


FIG. 8—Rat's phrenic nerve-diaphragm preparation. Maximal single shocks 5 per min. W=wash. (a) shows the depression caused by  $0.1 \mu\text{g}$  eserine on a fresh preparation (b) the effect of  $50 \mu\text{g}$  adrenaline 35 min later, (c) shows the augmentation caused by  $0.2 \mu\text{g}$  eserine in the same preparation 3 hours later, and the further increase due to  $50 \mu\text{g}$  adrenaline after eserine had been washed out. In (d)  $50 \mu\text{g}$  adrenaline was added without removing the eserine, note the similarity between (d) and (b).





FIG 9—Rats phrenic nerve-diaphragm preparation. Maximal single shocks 6 per min in a fresh preparation (a) adrenaline caused a depression in the presence of eserine, 3 hours later (b) it caused augmentation

as little as 0.1  $\mu$ g eserine was seen to cause depression (Fig 8a). This depression had not passed off 35 min later though the solution had been changed twice. When adrenaline was added a further depression was observed (Fig 8b). The preparation was now left for 4 hours, but in spite of washing, the height of contractions did not return to normal. At this stage, however, 0.2  $\mu$ g eserine produced a big rise in tension and, after it had been washed out, adrenaline caused a further rise (Fig 8c). After the solution had been changed, the contractions were allowed to return to their former level and 0.2  $\mu$ g eserine was once more added (Fig 8d) followed—(without wash)—by adrenaline which caused a depression similar to that of 4 hours previously in Fig 8b. The two opposite effects of adrenaline shown in Fig 8c and d in the presence of eserine correspond precisely to those shown in Fig 5a and b, in the presence of prostigmine.

There was no doubt that if the rat's diaphragm had been prepared for several hours and was stimulated continuously at a slow rate, its sensitiveness to the action of prostigmine and eserine gradually declined. With stimulation at 8 per min in a fresh preparation, 2  $\mu$ g prostigmine caused a short increase of muscle contractions followed by a depression. In another preparation, which had been working for several hours, 20  $\mu$ g prostigmine still produced a big increase which then gave way to depression. Similarly adrenaline is more likely to produce a muscular depression in the beginning of an experiment than at a later stage. In Fig 9a adrenaline following 0.2  $\mu$ g eserine caused a big depression, but 3 hours later as shown in Fig 9b, the same dose produced a big increase.

### 3 *The Action of Atropine, Curarine and Procaine*

The action of atropine has been studied both before and after prostigmine or eserine. In big doses atropine shows its well-known "curare-like" action. The contractions of the isolated rat's diaphragm were diminished by doses of 4–5 mg. atropine, a concentration of 1 in 20,000 to 1 in 25,000. Sometimes, however, a very slight transitory increase was seen before the gradual decline. With smaller doses of atropine up to 0.5 mg. no effect on normal muscle contractions could be seen, but after the addition of 1–2 mg. to the bath, making a concentration of 1 in 50,000 to 1 in 100,000, the curious observation was made that the muscle responses to maximal single shocks were increased as is shown in Fig 10a and 11a. There was never a sudden increase, but the effect was always gradual, at the most 20 per cent and more often less. This increase of normal muscle contractions was only obtained in fresh preparations.

The observation that eserine, 1 in 1,000 million, on a fresh preparation might cause a depression of muscle tension produced by maximal shocks (see Fig 8a) raised the question whether a fresh preparation was in a condition of mild acetylcholine paralysis? Dale and Gaddum (1930) observed that atropine antagonized the action of acetylcholine on the isolated denervated kitten's diaphragm. Brown (1937) showed that in the frog's gastrocnemius the injection

of 0.1 cc atropine sulphate, 1 in 1,000, abolished the muscle response to motor nerve stimulation and depressed, but did not abolish, the muscle response to a close intra-arterial injection of acetylcholine. On the other hand, Abdon (1940) found that those doses of atropine which abolished the muscle twitch caused by acetylcholine injected intra-arterially, did not have any influence on the muscle contractions provoked by motor nerve stimulation. In his experiments on frogs the muscle was immersed in atropine sulphate, 1 in 10,000, while in rabbits

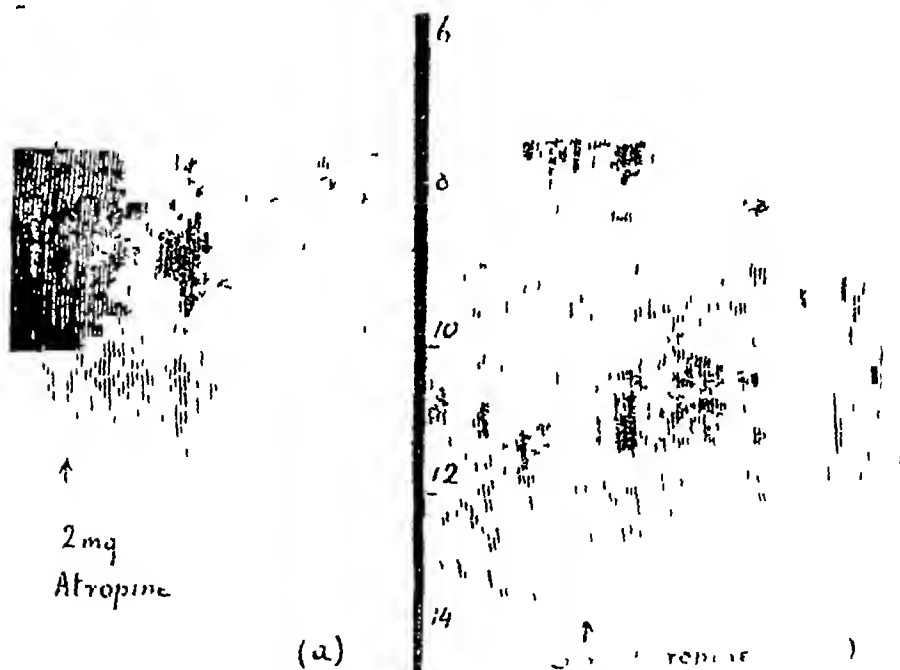


FIG 10—Rat's phrenic nerve-diaphragm preparation. Maximal single shocks 8 per min. The effect of atropine on (a) the contractions of a fresh muscle, (b) on the contractions increased by 0.2  $\mu$ g eserine.

10 mg/kg atropine sulphate was injected intravenously. Abdon suggested that authors who failed to abolish the effect of intra-arterially injected acetylcholine seemed to have used too small amounts of atropine. But Brown used 10 times as much as Abdon and the discrepancy of their findings cannot therefore be explained in this way.

In the isolated rat's diaphragm atropine caused a depression of muscle contractions in response to maximal nerve stimuli when they were increased by eserine or prostigmine. This is shown in Fig 10b. While the size of muscle contractions was still increasing, owing to the addition of 0.2  $\mu$ g eserine to the bath, 3 mg atropine reduced them to their original size.

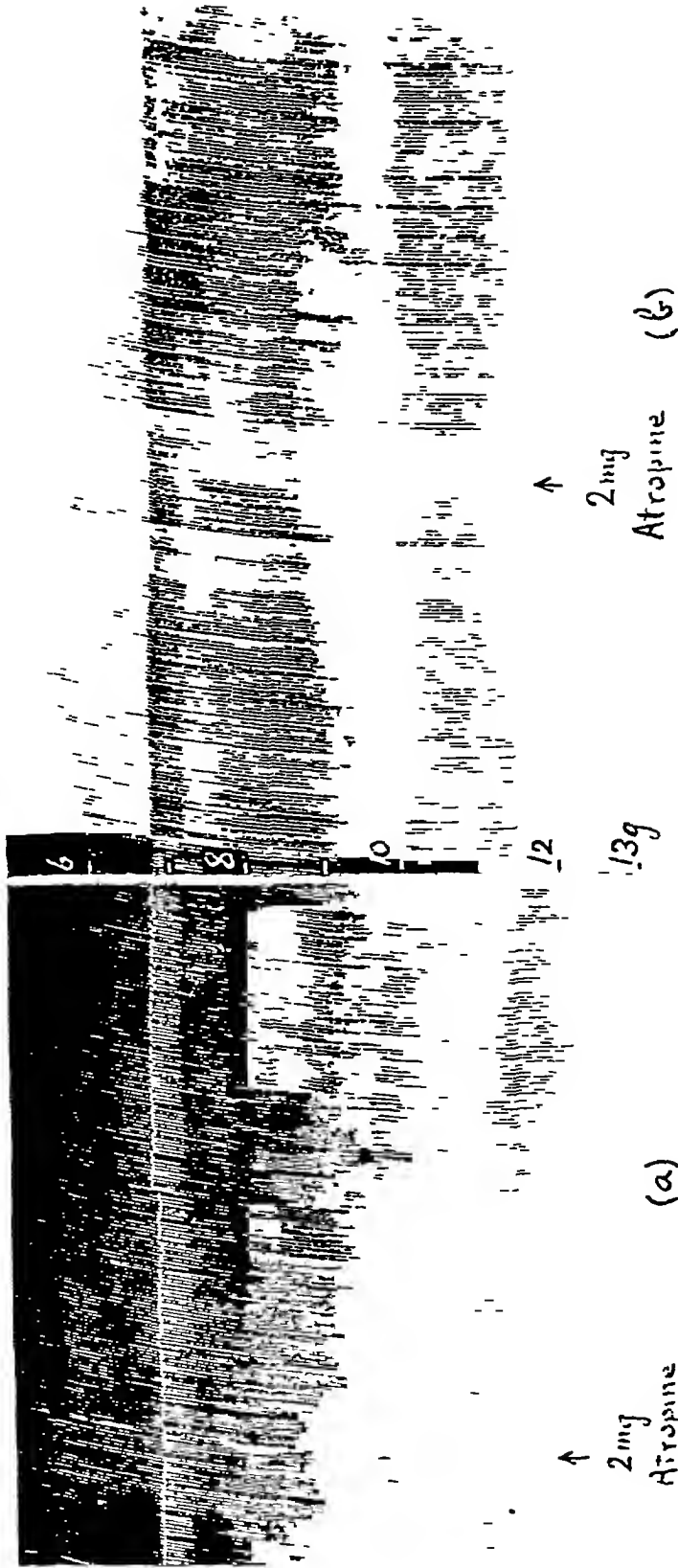


FIG 11—Similar to FIG 10. Maximal single shocks 9 per min. The effect of atropine on (a) the contractions of a fresh muscle, (b) on the contractions depressed by 20  $\mu$ g eserine

On the other hand, when the muscle contractions were depressed by an overdose of eserine, atropine augmented them. In Fig 11 is shown an experiment in which atropine, at the beginning of the experiment (Fig 11a), caused some increase of muscle contractions. The solution was changed several times and the preparation was stimulated continuously for 3 hours. After this period 20  $\mu$ g eserine was given and first increased the size of contractions, then depressed them below their original size. When now 2 mg atropine was added an augmentation was seen (Fig 11b). There is a striking similarity between the effects in Fig 10a and b, which suggests that in both instances atropine removes a depression which is due to an excess of acetylcholine.

Briscoe (1936) has shown that subparalytic doses of curarine restore muscle contractions previously depressed by prostigmine, and it seemed interesting to compare the action of atropine with that of curarine. The dependence of the action of prostigmine and of eserine on the frequency of stimulation was studied by Briscoe (1936) and by Bacq and Brown (1937). In Fig 12a is shown the effect of periods of fast stimulation on the normal muscle before and after 0.5  $\mu$ g eserine. Though at a rate of 5 per min the size of contractions was increased in the presence of eserine, faster stimulation (18 and 50 per min) caused a rapid decline, the steepness of which was directly proportional to the rate of stimulation. After the administration of larger doses of eserine (2  $\mu$ g in Fig 12b) while the nerve was stimulated at 17 per min muscle contractions declined rapidly below normal. The addition of 1 mg atropine stopped this decline and started a slow increase. Further 2 mg atropine had no more effect than to increase the overthrow of the lever, (this is due to the muscle relaxing more quickly). When 50  $\mu$ g curarine was added to the bath a sudden increase of contractions took place and now, in the presence of curarine, it was found that the relation between the size of muscle contractions and the rate of stimulation was reversed. At a slow rate the height of contractions declined, at a fast rate it increased. It is generally accepted that curarine raises the threshold of the muscle for acetylcholine. Therefore, when the rate of stimulation is slow and, in the presence of eserine, only small amounts of acetylcholine accumulate, the muscle contractions may decline below their maximal size, but with fast stimulation, when more acetylcholine accumulates, a normal height of contractions may be attained.

If one assumes that atropine has only a very slight threshold raising, curare-like action, this would explain why it was found to be more effective in antagonizing an eserine depression at slow rates of stimulation than at faster rates (compare Figs 11b and 12b). Fig 13 shows the muscle contractions of a preparation fully eserinized (100  $\mu$ g) and atropinized (4 mg). At a rate of 5 per min. contractions were bigger than at 18 or 50 per min. But even at this fastest rate no severe depression was seen. If atropine exerted its action only by raising the threshold of the muscle, like curarine but to a lesser degree, then 50 stimuli per min in the presence of an enormous dose of eserine should have depressed muscle contractions much further. One may therefore attempt to explain the

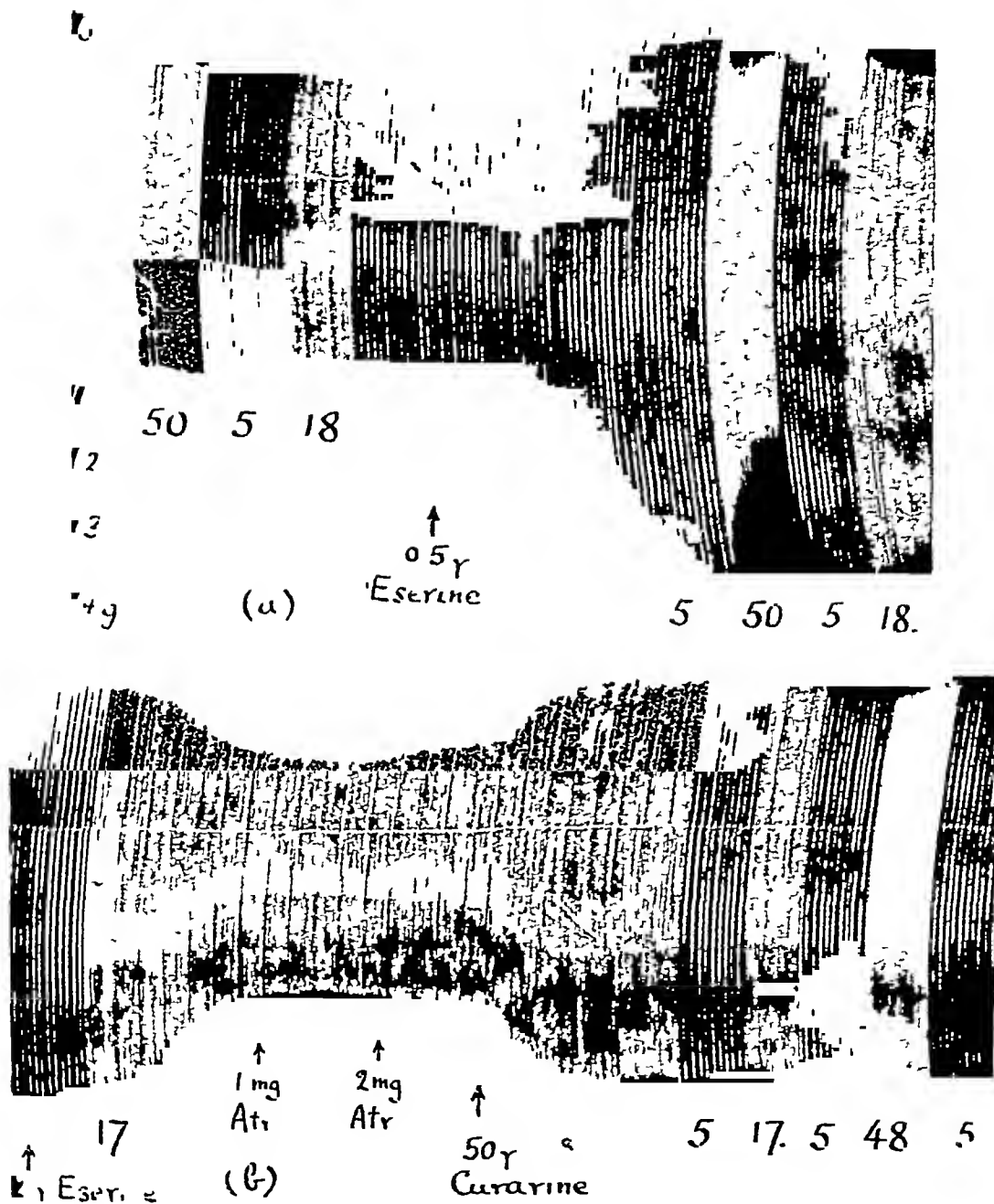


FIG 12—Rats phrenic nerve-diaphragm preparation. Maximal single shocks at rates per min indicated by figures. Note that after eserine (a) contractions decline during fast and recover during slow rates while after curarine (b) contractions decline during slow and increase during rapid rates.

action of atropine by assuming that atropine reduces the amount of acetylcholine formed. This possibility was first considered by Brown (1937).

Harvey (1939) showed that procaine suppressed the output of acetylcholine from the superior cervical ganglion during preganglionic stimulation. He suggested that in skeletal muscle procaine, besides acting like curare, also diminished the liberation of acetylcholine from the motor nerve endings. This view was

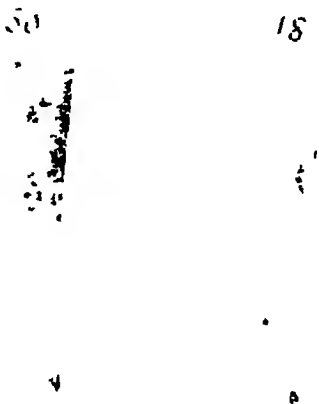


FIG. 13 —Maximal single shocks at 50, 5 and 18 per min. in a fully eserized and atropinized phrenic nerve-diaphragm preparation.

supported by Jaco and Wood (1944). Fig. 14 shows an experiment in which the effect of procaine was observed (a) during a prostigmine-augmentation, (b) during a depression after an overdose of prostigmine. The increased muscle contractions in (a) were reduced by procaine to their normal height. The diminished muscle contractions in (b) were also at first slightly reduced but then steadily increased although they did not reach their original size. Nevertheless, the gradual onset of this effect is similar to the records obtained with atropine.

It is possible to observe the effect of excess acetylcholine on the muscle contractions by increasing the rate of nerve stimulation in the presence of an anticholinesterase and also by adding acetylcholine to the bath. If atropine and procaine act like curarine by raising the threshold of the muscle to acetylcholine then the muscle response to acetylcholine released from the nerve and to acetylcholine introduced from outside should be affected in the same way. If, however, the activity of the nerve is affected by atropine and procaine, then in their presence the response of the muscle to the application of fast stimulation should differ from the response to the addition of acetylcholine to the bath.

In Fig. 15 (a) is shown the effect of fast stimulation on a muscle treated with prostigmine. The size of muscle contractions declined and a further depression

(a)

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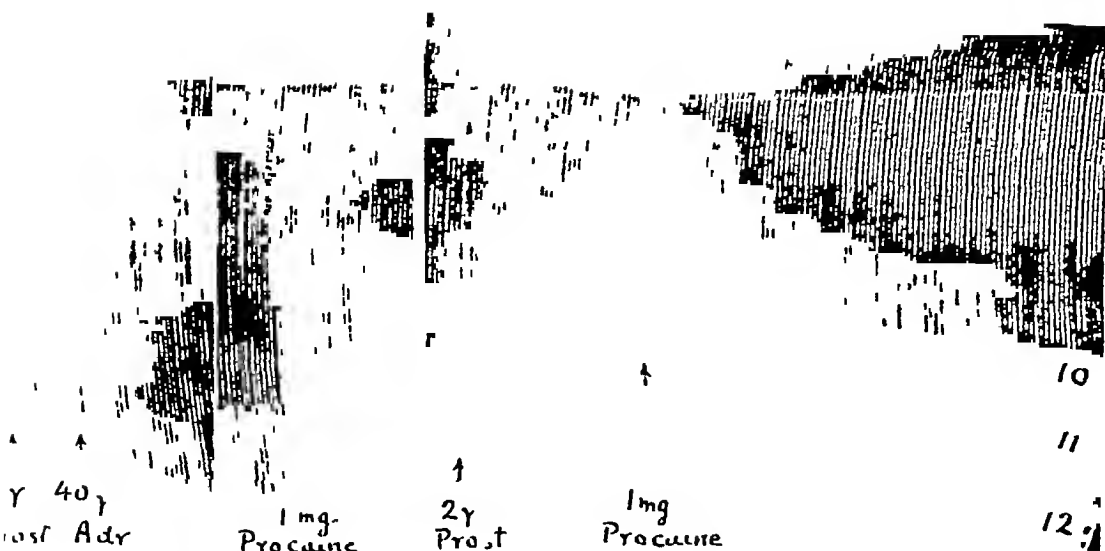
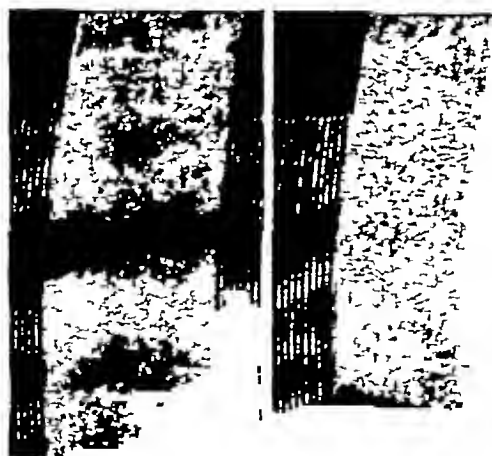


FIG 14—Rat's phrenic nerve-diaphragm preparation. Maximal single shocks 8 per min. The effect of 1 mg. procaine on the size of muscle contractions (a) when increased, (b) when depressed by prostigmine



(a)

(b)

FIG 15—Rat's phrenic nerve-diaphragm preparation. Maximal single shocks 5 per min, 2 μg. prostigmine present throughout. The effect of increasing the rate of stimulation to 28 per min and of 0.5 μg. acetylcholine (a) before and (b) after 35 μg. curarine.



was produced by the addition of 0.5 mg acetylcholine to the bath. In (b) after 35  $\mu$ g curarine the muscle contractions slowly increased during the period of faster stimulation and the addition of 0.5 mg acetylcholine did not interrupt this slow augmentation. As curarine raises the threshold of the muscle to acetylcholine neither the rapid stimulation nor the addition of 0.5 mg acetylcholine to the bath caused a depression. We know that in the presence of curarine the nerve liberates acetylcholine as before (Dale, Feldberg and Vogt, 1936) and the amount introduced into the bath remained the same. But the muscle, being less sensitive was not paralysed.

In Fig. 16 is shown a comparison between the action of atropine and curarine. Throughout 2  $\mu$ g prostigmine was added to the bath to ensure the accumulation

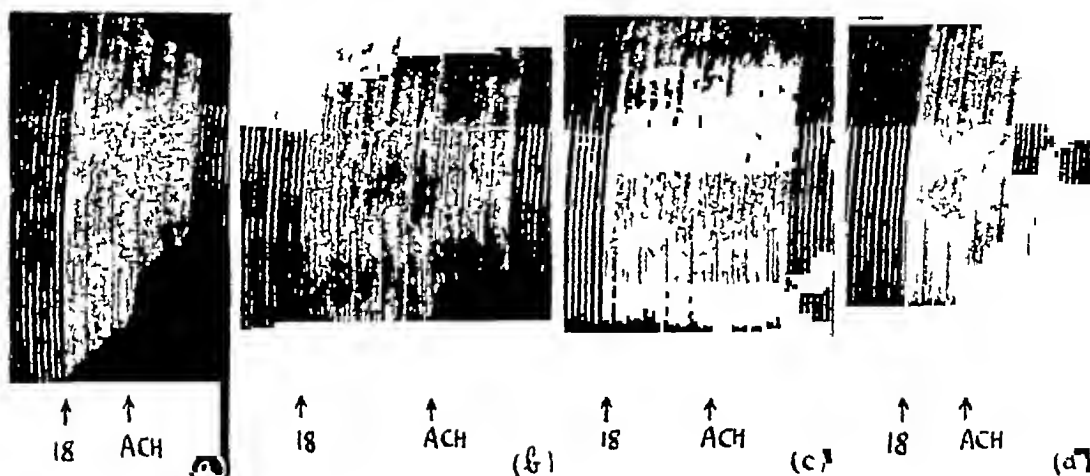


FIG. 16—Similar to Fig. 15. Maximal single shocks 6 per min. 2  $\mu$ g prostigmine present throughout. The effect of increasing the rate of stimulation to 18 per min., and of 0.5 mg acetylcholine (a) before and (b) after 2 mg atropine, (c) after 35 mg curarine (d) after 2 mg. atropine.

of acetylcholine. In (a) a depression was produced by fast stimulation and a further depression by additional acetylcholine. In (b) 2 mg atropine was added. The muscle contractions were at first slightly depressed during fast stimulation but steadily regained their original height, the addition of 0.5 mg acetylcholine caused a marked depression. After the preparation had been washed out several times during an interval of 20 min, 35  $\mu$ g curarine was added to the bath (c) this abolished both the depression due to rapid nerve stimulation and that due to added acetylcholine. After another interval in which the curarine was washed out the sequence was once more repeated in the presence of atropine (d).

The action of procaine was indistinguishable from that of atropine. This is shown in Fig. 17. Throughout 2  $\mu$ g prostigmine was present in the bath and a

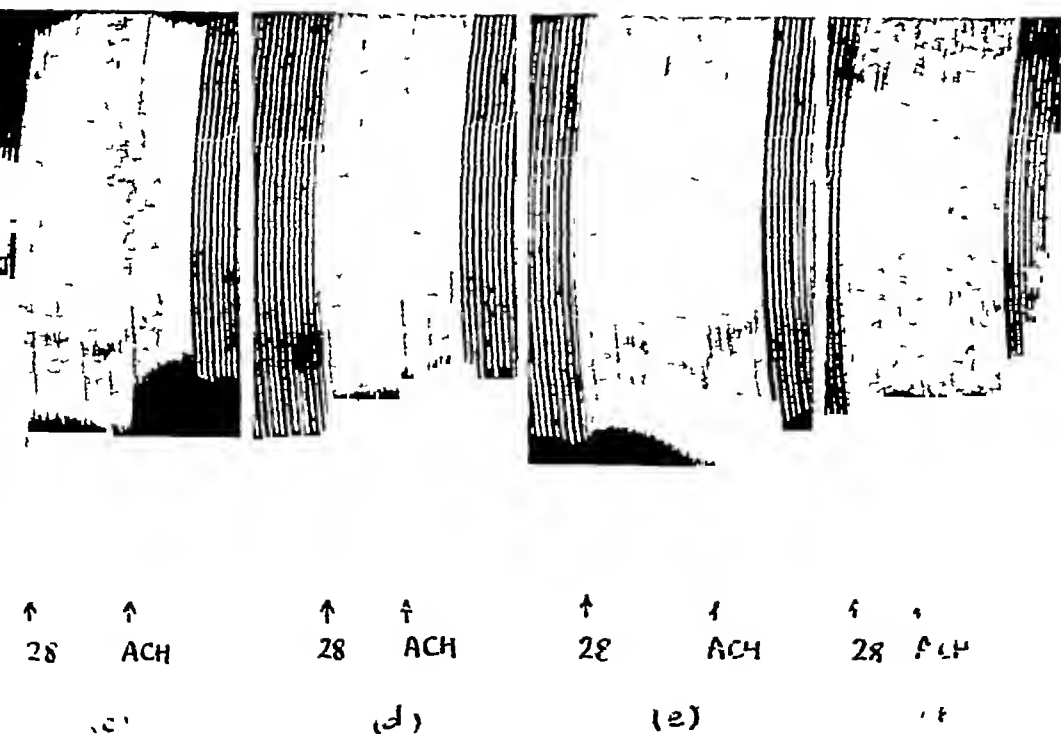
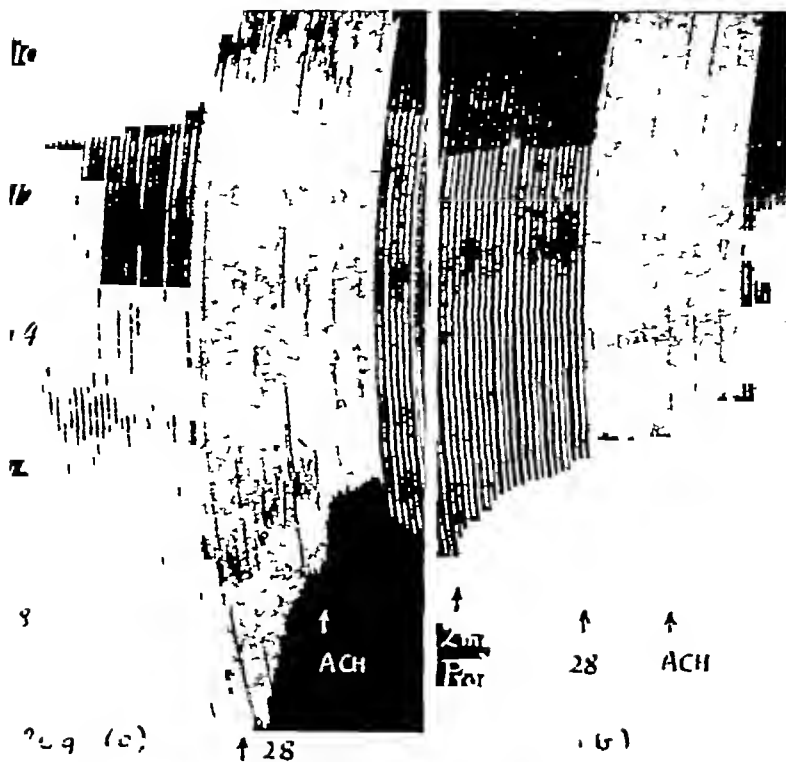


FIG 17—Similar to Fig. 15. Maximal single shocks 6 per min. 2  $\mu$ g. prostigmine present throughout. The effect of increasing the rate of stimulation to 28 per min., and of 0.5 mg. acetylcholine (a) before and (b) after 2 mg. procaine, (c) after washing out (d) after 2 mg. atropine, and (e) after washing out (f) after 35  $\mu$ g. curarine

depression was first produced by fast stimulation and secondly by additional acetylcholine. In the presence of 2  $\mu$ g prostigmine only (a) both effects were marked. After 2 mg procaine (b) and also after 2 mg atropine (d), muscle contractions at first declined with increased rate of stimulation but soon remained steady, whereas the addition of 0.5 mg acetylcholine caused a progressive depression. When the bath was changed, both after procaine (c) and after atropine (e), rapid stimulation caused an initial depression followed by a gradual increase of contractions, but added acetylcholine caused a profound decline. Finally, in the presence of 35  $\mu$ g curarine (f), both effects were abolished.

### DISCUSSION

The action of adrenaline was studied on an isolated mammalian nerve muscle preparation in order to exclude vascular effects. The results described in this paper confirm the conclusions drawn from previous experiments in which adrenaline was injected into the bloodstream. They leave no doubt that adrenaline has an action on the muscular response to nerve stimulation which is independent of its vasoconstrictor action.

It was found that muscle contractions in response to submaximal nerve stimuli are increased if adrenaline is added to the fluid in the bath in which the muscle is suspended. This increased muscle response is seen not only when the nerve is stimulated with open electrodes, in which the adrenaline may reach the site of nerve stimulation, but also when the nerve is stimulated with fluid electrodes, where the adrenaline cannot reach the point of stimulation. Adrenaline has no effect on the muscle response to maximal motor nerve stimuli. It causes an augmentation in the size of maximal contractions only when fatigue has occurred after prolonged maximal stimulation. This phenomenon is generally attributed to an improvement of neuromuscular transmission.

It is difficult to explain why in the non-fatigued preparation adrenaline increases the muscle response to submaximal stimuli whereas it does not affect maximal contractions. The results so far obtained with submaximal stimulation suggest that adrenaline acts by improving neuromuscular transmission, but further investigation is in progress. This explanation would be in agreement with observations on the perfused superior cervical ganglion (Bülbring, 1944) in which adrenaline improved synaptic transmission and thus increased the response of the nicotinic membrane to submaximal stimulation of the preganglionic nerve fibres. The effective concentration of adrenaline in the perfusion fluid of the ganglion was, however, 1 in 100 to 1 in 200 million, whereas in the fluid surrounding the isolated muscle it was 1 in 2 to 1 in 10 million. The discrepancy may be due to the different way in which adrenaline reaches the tissue, much less may be required if it is carried in the circulation than if it acts by diffusion from outside. Dale and Gaddum (1930) used an even stronger concentration of adrenaline, 1 in 75,000, which increased the action of acetylcholine on denervated muscle.

Another action on skeletal muscle was described by Bülbring and Burn (1942), who found that adrenaline augmented the action of eserine or prostigmine. It was possible that this was due to a vascular effect. But the results were interpreted as indicating that adrenaline lowered the threshold of the muscle, thereby potentiating the action of acetylcholine which accumulated in the presence of the anticholinesterase. This view is supported by the results obtained on the isolated preparation. If the phrenic nerve is stimulated with maximal shocks at slow rates the contractions of the diaphragm are increased by prostigmine or eserine and a further increase is produced by the addition of adrenaline to the bath. If the nerve is stimulated at more rapid rates or if the anticholinesterase is allowed to exert a more prolonged action, then adrenaline causes a depression of muscle contractions. Both observations indicate that adrenaline in the absence of vascular changes intensifies the effect on the muscle of an accumulation of acetylcholine.

In the isolated muscle, especially at the beginning of an experiment, the effects of excess acetylcholine are more readily observed than in a muscle with its normal circulation. This fact is reminiscent of conditions in the perfused superior cervical ganglion described by Brown and Feldberg (1936). They showed that in the perfused ganglion during continued maximal preganglionic stimulation the output of acetylcholine started at a high level and fell rapidly during the first 20-60 min. after which it continued at a steady low level. Consequently, when the perfusion fluid contained eserine, the effect of excess acetylcholine on the contraction maintained by the nictitating membrane during continued preganglionic stimulation differed in the initial stage from the effect later on. An increase in strength of stimulation, as well as the injection of acetylcholine into the perfusion fluid, at first caused a depression, but 44 min. later both produced an augmentation. The results obtained on the isolated striated muscle preparation suggest that similar conditions prevail. In the beginning of an experiment motor nerve stimulation seems to produce such a large amount of acetylcholine that the addition even of a small dose of an anticholinesterase may cause an accumulation sufficiently high to produce paralysis. The same dose of eserine, given later, may cause an increase of muscle contractions. Similarly, in a fresh preparation, after a dose of eserine which by itself may exert no action, adrenaline causes a depression, whereas several hours later the same dose of adrenaline causes augmentation.

It seems possible that when this isolated muscle is freshly prepared, acetylcholine accumulates in it even in the absence of an anticholinesterase. This hypothesis is supported by the observation that, in a fresh muscle, contractions were slightly augmented by the addition of atropine to the bath. This observation was puzzling until it was shown that atropine antagonized any effects of increased amounts of acetylcholine accumulated by nerve stimulation in the same way as it antagonizes the action of acetylcholine, eserine and prostigmine in the spinal cord (Bülbring and Burn, 1941). Thus, when muscle contractions were

increased by eserine or prostigmine, the addition of atropine reduced them to normal. On the other hand, when muscle contractions were decreased by an overdose of an anticholinesterase or by a faster rate of stimulation atropine counteracted the depression. In both instances atropine acts like curarine. A difference was, however, observed when excess of acetylcholine was not only produced by rapid nerve stimulation in the presence of eserine or prostigmine but also by addition of acetylcholine from outside. Curarine in the presence of an anticholinesterase prevented the muscular depression caused by fast stimulation and that caused by a dose of acetylcholine alike. But atropine did not prevent the depressant effect of a dose of acetylcholine added to the bath. The conclusion was therefore drawn that atropine interfered with the liberation of acetylcholine from the motor nerve ending rather than that atropine affected the muscle in the same way as curarine. Brown (1937) came to the same conclusion when he found that in frog's muscle atropine rendered nerve stimulation ineffective, leaving, however, a large part of the acetylcholine response still present.

In the phrenic nerve-diaphragm preparation the effects caused by atropine were very similar to those obtained with procaine. As procaine depresses the activity of sensory nerves a similar action on motor nerves is not surprising. It is well known that atropine possesses a weak local anaesthetic action. But while Harvey (1939) showed that procaine diminished the output of acetylcholine from the perfused superior cervical ganglion during preganglionic stimulation, Brücke (1937) showed that atropine increased the output of acetylcholine in the frog's heart during vagus stimulation. The amount of acetylcholine escaping into the perfusion fluid may not be a true measure of the amount liberated from the nerve, but may be modified by the amount which is capable of or prevented from combining with the receptive substance. Recently Dawes (1946) has shown that atropine and procaine in large dosage exert a similar action on heart muscle, i.e. both of them prolong the refractory period in the same way as quindine.

#### SUMMARY

1 A strip of the rat's diaphragm with the phrenic nerve can be used as an isolated mammalian nerve muscle preparation.

2 If single submaximal shocks are applied to the phrenic nerve the contractions of the diaphragm muscle are increased by the addition of adrenaline to the bath. This effect is observed whether the adrenaline reaches the site of nerve stimulation or not. Unless the muscle is fatigued, adrenaline has no effect on the muscle response to maximal nerve stimuli.

3 The muscle contractions elicited by maximal nerve stimuli are increased by small doses of eserine or prostigmine at slow rates of stimulation. A depression is produced by prolonged action or by an overdose of the anticholinesterase or by increasing the stimulation rate in the presence of the anticholinesterase.

4 Adrenaline augments the action of eserine and prostigmine. This may be

observed as an increase or as a decrease in the size of muscle contractions according to various conditions. Muscle contractions are increased when the amount of the anticholinesterase is small and when the rate of stimulation is slow. The size of contractions is depressed by adrenaline after an overdose of the anticholinesterase or with faster rates of stimulation.

5 The depressant effect of eserine or prostigmine and of subsequent adrenaline is more readily observed in a fresh preparation than in one which has been stimulated for several hours. The possibility that acetylcholine may accumulate during the initial stages of motor nerve stimulation in this isolated preparation is discussed.

6 Atropine increases slightly the size of muscle contractions elicited by maximal nerve stimuli in a fresh preparation.

7 If muscle contractions are increased by eserine or prostigmine, atropine reduces them to their normal size. If muscle contractions are depressed by an overdose of an anticholinesterase or by a faster rate of stimulation then atropine counteracts the depression.

8 In the presence of an anticholinesterase curarine abolishes the depressant effects of excess acetylcholine, whether this be produced by rapid nerve stimulation or by adding acetylcholine to the bath. Atropine differs from curarine. It abolishes the depressant effect of rapid motor nerve stimulation but it does not abolish the depression caused by the addition of acetylcholine to the bath.

9 The action of atropine was found to be very similar to that of procaine. The conclusion was therefore drawn that atropine acts not only by raising the threshold of the muscle, as curarine does, but also by interfering with nervous activity.

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# THE EFFECT OF ETHANOL, METHANOL, PARALDEHYDE AND ACETONE ON THE PRESSURE OF THE CEREBROSPINAL FLUID OF THE DOG

BY

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Evidence has been presented in an earlier paper (Bedford, 1941), that ether dilates the blood vessels of the brain and consequently raises the pressure of the C.S.F. In the present series of experiments, a study has been made of the effect of ethanol and certain related compounds on the pressure of the C.S.F.

*Experimental Procedure*—Dogs were anaesthetized with sodium iso-amyl ethyl barbiturate (Amytal, Lilly), administered intravenously. Details of the technique are given in an earlier paper (Bedford, 1938). After anaesthesia was complete, a tube with side-valve was tied into the trachea. In all experiments, a minimal degree of pulmonary ventilation was maintained by means of a pump. The pressure of the C.S.F. was recorded from the cisterna magna by means of a vertical glass manometer of 1 mm. bore, readings of the pressure were taken at minute intervals. The mean arterial pressure was recorded from a cannula in the femoral artery.

Special attention was paid to the action of ethanol, some experiments were, however, performed with methanol, paraldehyde or acetone.

In one group of experiments, the dogs were made to inhale air containing varying amounts of the vapour of the liquid under investigation. This was effected by placing a reservoir containing the undiluted liquid between the respiratory pump and the animal, the air from the pump bubbled through the liquid. By means of a simple device, it was possible to vary at will the proportion of air from the pump which passed through the liquid. No absolute measurements of concentrations could be made. The reservoir was immersed in a water bath at 40° C., the tubing from the reservoir to the animal was also heated over the greater part of its course.

In another series of experiments, the effect of intravenous administration was studied. The liquid in 10 per cent (v/v) concentration in isotonic NaCl solution, was introduced at a measured rate into a tributary of the saphenous vein. The duration of an average experiment was 1½ hours.

## RESULTS

### *The Effect of Ethanol on the Pressure of the C.S.F.*

(a) *Inhalation*—The effect of inhalation of ethanol was studied in five dogs. No change was noticed in the pressure of the C.S.F. until a fall occurred in the mean arterial B.P. An immediate fall in the pressure of the C.S.F. was then observed, this fall was directly proportional to the fall in B.P. The pressure of

the C.S.F. and the mean arterial B.P. rose simultaneously to their original levels when the concentration of the ethanol vapour was reduced. Apart from changes resulting from a fall in B.P., the results were the same when ethanol was administered suddenly in high concentration and when dogs were made to breathe a low concentration of ethanol vapour which was gradually increased to maximum tolerance. Abrupt cessation of administration of ethanol was also unaccompanied by any change in the pressure of the C.S.F. The results of a typical experiment are illustrated in Fig. 1.

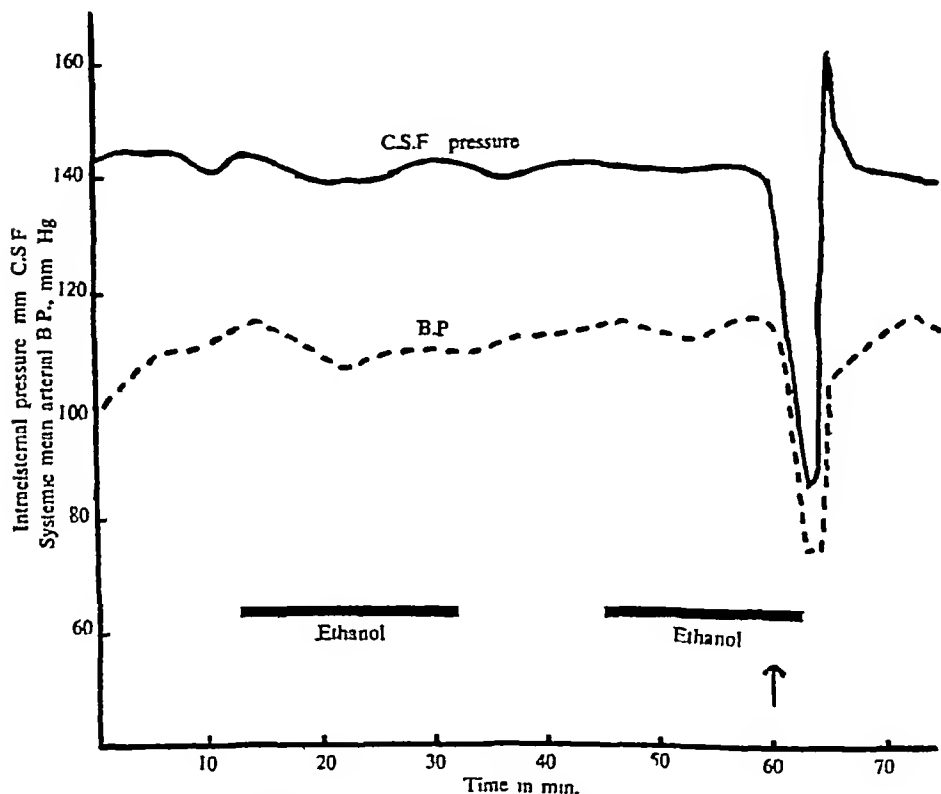


FIG 1—The effect of inhalation of ethanol on the pressure of the C.S.F. The concentration of ethanol vapour during the first period of administration failed to cause a fall in B.P. During the second period of administration, the ethanol vapour was increased at the arrow, to a concentration that caused a fall in B.P. The temporary rise in C.S.F. pressure to a level higher than its original level, which followed the replacement of ethanol vapour by air, is not specific for ethanol but is generally found to accompany any sudden rise in systemic B.P.

(b) *Intravenous Administration.*—The intravenous administration of ethanol was studied in six dogs. A 10 per cent (v/v) concentration of ethanol in isotonic NaCl solution was introduced at a rate of 2 ml per min., a greater rate than this usually produced a rapid fall in blood pressure. The maximum



volume of this solution administered during a single experiment was 96 ml, it was introduced over a period of 48 min into a dog of 10 kg. The concentration of ethanol in the blood would be well in excess of that needed to cause coma in man. No change was noticed in the pressure of the cerebrospinal fluid except in those instances in which the alcohol produced a fall in B.P. The accompanying fall in the pressure of the C.S.F. was clearly dependent on the fall in blood pressure.

#### *The Effect of Methanol, Acetone and Paraldehyde on the Pressure of the C.S.F.*

Inhalation of methanol (4 dogs), paraldehyde (8 dogs) and acetone produced effects on the B.P. and C.S.F. very similar to those described above for ethanol. The effect of intravenous administration of paraldehyde (10 per cent v/v in isotonic NaCl) was more dramatic than that observed with ethanol, a profound fall of arterial B.P. occurring when the solution was introduced at a rate exceeding 1 ml/min (3 dogs). The effect of intravenous administration of methanol was indistinguishable from that of ethanol (2 dogs).

#### SUMMARY

It would appear from the above experiments that ethanol, methanol, paraldehyde and acetone have no immediate influence on the pressure of the C.S.F. unless they are administered in amounts sufficient to produce a fall of systemic B.P., a fall then occurs in the pressure of the C.S.F. which is directly dependent on the fall in B.P. The same results were obtained whether ethanol, methanol and paraldehyde were administered by inhalation or intravenously, acetone was only administered by inhalation. It is concluded that any direct action these compounds may have on the cerebral blood vessels is insignificant in character.

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# CHEMOTHERAPEUTIC ACTION OF AMIDINE AND PHENANTHRIDINIUM COMPOUNDS IN *T CONGOLENSE* INFECTIONS

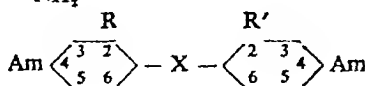
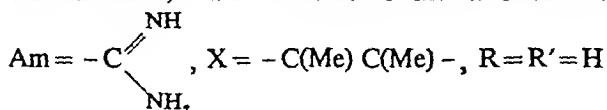
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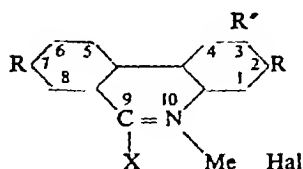
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In evaluating the therapeutic activity of a series of diamidine compounds we found that while certain of them (particularly nuclear halogen-substituted derivatives of stilbamidine) were highly active against *Trypanosoma equiperdum* and *T rhodesiense*, few were curative in *T congolense* infections. One of the most active was 4,4'-diamidino- $\alpha\beta$ -dimethylstilbene (991 or 'dimethyl stilbamidine'). Its structure is shown from the general formula I, where



I



II

The activity of this compound has been described in laboratory animals by Fulton & Warrington Yorke (1943, a and b) and in cattle by Carmichael & Bell (1943, 1944) and Daubney & Hudson (1943). It was found active in field trials but the therapeutic index was small; there is usually a narrower margin of safety in large animals than in small laboratory animals.

Recently certain phenanthridinium compounds have been found highly active against *T congolense*, with a greater therapeutic index than that of the amidines (Walls, 1945; Browning, Morgan, Robb and Walls, 1938; Hornby, Evans and Wilde, 1943; Carmichael and Bell, 1943; Browning and Calver, 1943, and Bell, 1945). The phenanthridinium compounds have the general formula II.

The most active were 897, 7-amino-9-*p*-aminophenyl-10-methylphenanthridinium chloride ( $\text{R} = \text{NH}_2$ ,  $\text{R}' = \text{R}'' = \text{H}$ ,  $\text{X} = \text{---} \text{C}_6\text{H}_4\text{---NH}_2$ ), and 1553, 2,7-diamino-9-phenyl-10-methylphenanthridinium bromide ( $\text{R} = \text{R}' = \text{NH}_2$ ,  $\text{R}'' = \text{H}$ ,  $\text{X} = \text{---} \text{C}_6\text{H}_5$ ).

Although dimethyl stilbamidine had been shown to be less promising in field trials than the phenanthridinium compounds, we were interested in carrying the

comparison further in the laboratory, since the early experiments had been conducted with an acute strain in mice, whereas in cattle the disease takes a chronic course. This paper is therefore concerned partly with a comparison of dimethyl stilbamidine and the phenanthridinium compound 897 against different strains of *T. congolense*, and also with the examination of other diamidine and phenanthridinium compounds, including some observations on the pharmacological actions of the compound 1553. The results have clarified to some extent the relationship between chemical structure and activity against *T. congolense*, but no correlation of activity has been found between this infection and *T. equiperdum rhodesiense* or *brucei* infections. Diamidines which were highly active against the latter trypanosomes were ineffective against *T. congolense*, and conversely, phenanthridinium compounds which were highly active against *T. congolense* showed little activity against *T. equiperdum*.

### METHODS

Subinoculations of each strain were made from infected mice by withdrawing heart blood, diluting appropriately with serum (normal horse serum 20 per cent)—citrate-saline, and injecting 0.3 cc of the suspension intraperitoneally into each mouse. This was carried out at the acute stage when trypanosomes were numerous in the peripheral blood (3 or 4+), 1+ = 1–10, 2+ = 20–30 trypanosomes per microscopic field, and so on. Groups of albino mice from an inbred laboratory strain were used, and only those were selected which after some days showed a moderate degree of parasitaemia (up to 2+). Solutions of the drugs were given by intravenous injection into the tail vein or subcutaneously; the latter method was more frequently used, since by this route the therapeutic index is almost always greater. Wet smear preparations of blood from the tail were examined periodically, a mouse was regarded as negative if no trypanosomes were found in at least 20 microscopic fields. Evaluation was made by determining the E.D. 50 (50 per cent of the animals cleared of trypanosomes from the peripheral blood within seven days), C.D. 50 (50 per cent of the animals free of trypanosomes for at least 28 days, 42 days for chronic strains), and L.D. 50 (average lethal dose). The calculations were made either by Litchfield and Fertig's method (1941) or by Irwin and Cheeseman's (1939) modification of Kärber's method. Where required the standard errors of these results are given. The chemotherapeutic index was determined by dividing the L.D. 50 by the C.D. 50, which is a more exact ratio than the usual ratio M.L.D./M.C.D. A similar method of assaying trypanocidal activity has recently been employed by Chen, Geiling and MacHattie (1945). The five different strains of *T. congolense* were utilized only in the comparative experiments with dimethyl stilbamidine and phenanthridinium compound 897, in other experiments only the acute strain III was used.

The strains of *T. congolense* employed were essentially of two types, namely those which produced acute and chronic infections in mice. Acute infections in mice may be fatal in about six days, whereas in cattle the chronic infection persists for a considerable time. The virulence in mice is often enhanced by passage, but in cattle the reverse may occur. The size of inoculum influences the onset of the infection, heavy inocula hastening the appearance of trypanosomes in the peripheral blood, although the effect is not very marked. The route of administration has a similar effect, intraperitoneal injection causing a quicker appearance than the subcutaneous route.

### Strains of *T. Congolense*

*I and II*—These two strains have been employed by Browning and Calver (1943), and Prof. C. H. Browning kindly supplied both strains. Strain I was described earlier by Brown-

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ing, Cappell and Gulbransen (1934), and produces a chronic infection with a relapsing course. Since it was originally obtained from Dr C M Wenyon it was probably derived from the same source as strain V. We confirmed the chronic nature of strain I and found that parasites appeared more quickly in the blood after intraperitoneal (four days) than after subcutaneous injection (five to eight days). After the first appearance of the parasites they increased usually to about 3+ within three to four days, although sometimes a longer period was observed. A negative phase followed, in which trypanosomes were not found in the peripheral blood, a period which might last for four weeks, followed by a relapse when numerous trypanosomes could be found. Subinoculations were made at the first appearance at the acme stage, i.e., at the height of the infection, as described by Browning. We confirmed that subinoculations made at other times altered the character of the infection. The majority of the mice survived this chronic infection, which corresponds to the condition found in cattle, in which the host may harbour trypanosomes for very long periods without succumbing.

Strain II produces an acute infection, the parasites becoming numerous in the blood and persisting until the death of the animal after a period varying from a few days to several weeks. It is not so virulent as strains III or IV. Prof C H. Browning has stated that it was received in a guinea pig in 1933 from Hornby in Tanganyika, its previous history is unknown.

III—This strain, kindly supplied by Dr J D Fulton, runs an acute course in mice, and we found it very constant in its virulence. After subinoculation trypanosomes appeared in the blood within two to four days, and all the mice died within seven to nine days. When the passage was made by subcutaneous injection these times were lengthened somewhat, the blood being full of parasites within nine days, and the mice dying within twelve days.

IV—This strain was kindly supplied by Dr F Hawking, who originally obtained it from the Liverpool School of Tropical Medicine. As would be expected, the course of the infection and its virulence were very similar to those of the parent strain III.

V—This strain was kindly supplied by Mr L. G Goodwin. After subcutaneous injection of peripheral blood no parasites are usually seen until eight to ten days. They increase up to fourteen days, and then decrease until at eighteen days none may be found. Subsequently relapses occur and the process may be repeated, the majority of the mice surviving for a long period. Subinoculations were always made at the first appearance of the parasites. In maintaining this strain for these experiments we used heart blood and subinoculated intraperitoneally. By this method and the use of a heavy inoculum it was found that the trypanosomes appeared within four days, and the acme stage was reached within five to eleven days, the parasites disappeared from the blood three days later, with subsequent relapses. The course of the infection showed considerable variation in different animals, and in this respect was entirely different from the acute strains. This factor also made this strain more difficult to work with when assessing the value of drugs. Only treatment which was effective within, say, seven days, could be attributed to the drug since spontaneous cures would occur later with temporary disappearance of trypanosomes.

## RESULTS

The compounds\* examined are given in the following list

No	Name of Compound
(a) Diamidines (i) Stilbene Series	
744	4 4'-diamidinostilbene
1311	2-methyl-4 4'-diamidinostilbene

\* All the compounds in (a) were dihydrochlorides, except 1126, which was the dilactate. Compounds 744 to 1126 of the stilbene series were prepared by Ashley and Harris (1946).

No	Name of Compound
(a) <i>Diamidines</i> (i) <i>Stilbene Series</i> (continued)	
1350	2 2'-dimethyl-4 4'-diamidinostilbene
1032	2-methoxy-4 4'-diamidinostilbene
1011	2-hydroxy-4 4'-diamidinostilbene
1129	2 2'-dihydroxy-5 5'-diamidinostilbene
1118	2-iodo-4 4'-diamidinostilbene
1015	2-amino-4 4'-diamidinostilbene
1025	2-acetamido-4 4'-diamidinostilbene
1126	4 4'-di-N-ethylaminostilbene
1005	4 4'-diamidino- $\alpha$ -methylstilbene
991	4 4'-diamidino- $\alpha\beta$ -dimethylstilbene
(ii) <i>Diphenoxy Series</i>	
782	4 4'-diamidino- $\alpha$ $\gamma$ -diphenoxypropane
1146	2-bromo-4 4'-diamidino- $\alpha$ $\gamma$ -diphenoxypropane
1272	2-bromo-4 4'-diamidino- $\alpha$ $\epsilon$ -diphenoxypentane
993	4 4'-diamidino- $\alpha$ $\eta$ -diphenoxyhexane
(iii) <i>Miscellaneous diamidines</i>	
1150	Phenanthrene-3 6-diamidine
1346	3 6-diamidinocarbazole
938	4 4'-diamidinodiphenylamine
(b) <i>Phenanthridinium Compounds</i>	
897	7-amino-9- <i>p</i> -aminophenyl-10-methylphenanthridinium chloride
1553	2 7-diamino-9-phenyl-10-methylphenanthridinium bromide
1355	7-amidino-9 - <i>p</i> - amidinophenyl - 10 - methylphenanthridinium chloride
1325	3-amino-9-(3'-pyridyl)-phenanthridine monomethochloride
1324	7-amino-9-(3'-pyridyl)-phenanthridine monomethochloride
193	9-( <i>p</i> -dimethylaminostyryl)-phenanthridine methochloride
196	7-acetamido-9-( <i>p</i> -dimethylaminostyryl)-phenanthridine methochloride
197	7-amino - 9 - ( <i>p</i> - dimethylaminostyryl) - phenanthridine methochloride
198	2-methyl - 9 - ( <i>p</i> - dimethylaminostyryl) - phenanthridine methochloride

The toxicity data of the three compounds examined in detail,

991	4 4'-diamidino- $\alpha\beta$ -dimethylstilbene dihydrochloride
897	7-amino-9- <i>p</i> -aminophenyl-10-methylphenanthridinium chloride
1553	2 7-diamino-9-phenyl-10-methylphenanthridinium bromide

are given in Table I

TABLE I  
Toxicity to Mice of 991, 897 and 1553

Compound	Injection	No of Mice	L.D. 50 (mg./g.)	Limits % (p = 95)
991	i.v.	40	0.049	85-118
	s.c.	40	0.198	83-121
897	i.v.	60	0.015	89-112
	s.c.	70	0.094	91-110
1553	i.v.	80	0.020	88-114
	s.c.	80	0.061	90-111

In Table II (i-v) the results are given of the comparative activities of 991 and 897 against five different strains (acute and chronic) of *T. congolense*

TABLE II  
Comparison of activity of 897 and 991 against different strains of *T. congolense*  
Infection in mice, 1-20 trypanosomes/microscopic field  
Cure disappearance of trypanosomes for at least 28 days  
Administration of compounds subcutaneously

Dose (mg /g.)	No of Mice	897	991
		No of Mice Cured	
(i) Strain I			
0.02	10	10	8
0.01	10	6	6
0.005	10	8	6
0.0025	10	6	2
0.00125	10	4	0
C.D 50 (mg /g.)		0.0027	0.0057
Limits % (p = 95)		58-173	58-171
Therapeutic index		35	35
(ii) Strain II			
0.02	10	10	10
0.01	10	8	10
0.005	10	10	6
0.0025	10	6	2
0.00125	10	4	0
C.D 50 (mg./g.)		0.0020	0.0041
Limits % (p = 95)		63-160	63-160
Therapeutic index		46	49
(iii) Strain III			
0.04	10	9	8
0.02	10	8	8
0.01	10	5	1
0.005	10	4	0
C.D 50 (mg./g.)		0.0090	0.0162
Limits % (p = 95)		60-167	60-167
Therapeutic index		10	12

(Continued overleaf)

Dose (mg./g.)	No of Mice	897	991
		No of Mice Cured	
(iv) Strain IV			
0.04	10	9	10
0.02	10	5	4
0.01	10	4	1
0.005	10	0	0
C.D 50 (mg./g.)		0.0157	0.020
Limits % ( $p = .95$ )		59-169	61-163
Therapeutic index		6	10
(v) Strain V			
0.04	10	8	9
0.02	10	6	10
0.01	10	1	5
0.005	10	2	4
C.D 50 (mg./g.)		0.0151	0.0078
Limits % ( $p = .95$ )		59-171	61-164
Therapeutic index		6	25

Table III shows the results of the evaluation of the activity of 1553 against an acute strain (III) of *T. congolense*

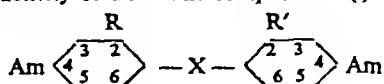
TABLE III

Evaluation of the activity of 2, 7-diamino-9-phenyl-10-methylphenanthridinium bromide (1553) against *T. congolense*, Strain III

Dose (mg /g.)	No of Mice	No of Mice cleared of Trypanosomes	
		3-7 Days	28 Days
0.001	10	10	10
0.0005	10	10	6
0.00025	10	9	2
0.000125	10	9	1
0.00008	10	8	—
0.000051	10	5	—
0.000032	10	3	—
0.00002	10	0	—
E.D 50 (mg /g.)		0.00005	
Limits % ( $p = .95$ )		72-139	
C.D 50 (mg /g.)		0.0004	
Limits % ( $p = .95$ )		61-165	
Therapeutic index		152	

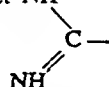
The results on the diamidines, phenanthrenes and phenanthridinium compounds examined (28 compounds in all) are given in Tables IV, V and VI. The compounds were injected subcutaneously and the mice were infected with *T. congolense*, strain III.

TABLE IV  
Toxicity and activity of diamidine compounds. (i) Stilbene series.



No	Substituent Groups			L.D. 50 (mg./g.)	E.D. 50 (mg./g.)	C.D. 50 (mg./g.)	Index
	X	R	R				
744	—CH CH—	H	H	0.18	0.02	0.08	2.3
1311	Do	Me	H	0.146	0.01	0.03	4.9
1350	Do	Me	Me	0.140	0.015	Not curative	—
1032	Do	OCH <sub>3</sub>	H	0.100	0.015	Do	—
1011	Do	OH	H	0.137	0.025	Do	—
1129	*Do	OH	OH	0.095	Inactive	—	—
1118	Do	I	H	0.332	0.025	Not curative	—
1015	Do	NH <sub>2</sub>	H	0.075	0.015	0.038	2.0
1025	Do	NH.Ac	H	0.150	0.008	0.038	4.0
1126	**Do	H	H	0.180	0.04	Not curative	—

\* Am = 5 5' \*\* Am = Et NH



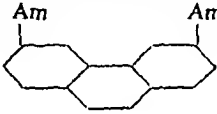
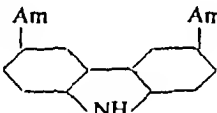
When the activities of 897 and 991 were compared the results showed that against the acute strains both compounds had approximately the same therapeutic indices. 897 was about twice as active and twice as toxic. The level of significance was taken at two to three times the standard error. The same result was obtained with the chronic strain I, but with strain V the compound 897 was only about half as active and consequently had a poorer therapeutic index. The difference, however, was not highly significant and only one comparison was made. The results with the phenanthridinium compound 1553 (Table III) showed quite clearly that it was significantly very much more active than either its analogue 897 or the diamidine 991.

It will be observed from Tables IV, V and VI that although a number of compounds showed activity, only 12 had a curative action, and eight of these—namely, 744, 1311, 1015, 1025, 1005, 1146, 1325 and 197 had therapeutic indices which were too small to be of interest. The activities of the phenanthridinium compounds 897 and 1553 and of the diamidine 991 have already been considered. It is noteworthy that phenanthrene-3,6-diamidine, 1150, displayed activity of a fairly high order.



TABLE V

Toxicity and activity of diamidine compounds (ii) Stilbene and diphenoxo series.  
X, R and R' as in Table IV

No	Substituent Groups			L D 50 (mg /g.)	E.D 50 (mg /g )	C.D 50 (mg /g )	Index
	X	R	R'				
1005	— C(Me) CH —	H	H	0.12	0.01	0.04	3.0
991	— C(Me) C(Me) —	H	H	0.198	0.004	0.007-0.016	12-28
938	— NH —	H	H	0.050	0.01	Not curative	—
782	— O — (CH <sub>2</sub> ) <sub>3</sub> — O	H	H	0.055	Inactive	—	—
1146	Do	Br	H	0.107	0.01	0.05	2.1
1272	— O — (CH <sub>2</sub> ) <sub>4</sub> — O	Br	H	0.23	Inactive	—	—
993	— O — (CH <sub>2</sub> ) <sub>7</sub> — O	H	H	0.063	Do	—	—
1150				0.160		<0.01	>16
1346				0.065	0.01	Not curative	—

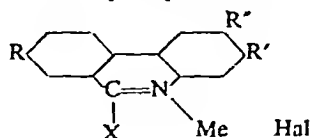
#### OBSERVATIONS ON THE PHARMACOLOGICAL PROPERTIES OF THE PHENANTHRIDINIUM COMPOUND 1553

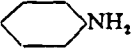

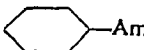



##### Action on Respiration

Respiration was recorded by Gaddum's method. In rabbits anaesthetized with urethane the intravenous injection of 5 or 10 mg of 1553 produced a marked fall in the depth of respiration, with little alteration in the rate, accompanied by a fall in blood pressure (Fig 1) the effects were transitory. The analogue 897 had a greater effect and the initial fall of blood pressure was followed by a large secondary rise. These effects were not reflex in origin since they occurred after vagotomy and denervation of the carotid sinus. There was some anoxaemia during the rise of blood pressure the arterial blood was noticed to become darker and there was a slight fall in the alkali reserve. When the injections were made into the right carotid artery the depressant effect on the respiration was markedly less, and the subsequent rise of blood pressure did not occur. With a sufficiently large dose death was caused by respiratory failure, this was noticed particularly with compound 897 the depressant effect of which on the respiration was more pronounced. In cats anaesthetized with either chloralose pentobarbitone, or

urethane, and in decerebrate preparations, the respiration was much less affected than in rabbits. In some preparations there was little alteration, or even an initial slight increase, the greatest depression of the respiration was observed in urethanized animals.

TABLE VI  
Toxicity and activity of phenanthridinium compounds



No	Substituent Groups				L.D. 50 (mg./g.)	E.D. 50 (mg./g.)	C.D. 50 (mg./g.)	Index
	X	R	R'	R''				
897	—  NH <sub>2</sub>	NH <sub>2</sub>	H	H	0.094	0.0025	0.009	10.4
1553	— 	NH <sub>2</sub>	NH <sub>2</sub>	H	0.061	0.00005	0.0004	152
1355	—  Am	Am	H	H	0.046	Inactive	—	—
1325	—  N	H	H	NH <sub>2</sub>	0.15	0.02	0.06	2.5
1324	—  N	NH <sub>2</sub>	H	H	0.174	Inactive	—	—
193	—CH—CH—  N(CH <sub>2</sub> ) <sub>1</sub>	H	H	H	0.004	Do	—	—
196	Do	NHAc	H	H	0.200	Do	—	—
197	Do	NH <sub>2</sub>	H	H	0.035	0.01	0.02	1.8
198	Do	H	CH <sub>3</sub>	H	0.003	Inactive	—	—

#### Action on Circulation

**Blood Pressure**—The general effect in anaesthetized rabbits and cats was a transitory fall in blood pressure, which was sometimes followed by a secondary

rise, especially with large doses, in urethanized rabbits. The compound 897 had a greater depressant action than 1553 in cats, the administration of a full dose of atropine reduced the fall in blood pressure but did not abolish it. The secondary rise in blood pressure may have been partly due to the anoxaemia, since it did not occur when the injections were given intra-arterially, and the effect on the respiration was not so marked. In rabbits ergotoxine did not abolish the rise

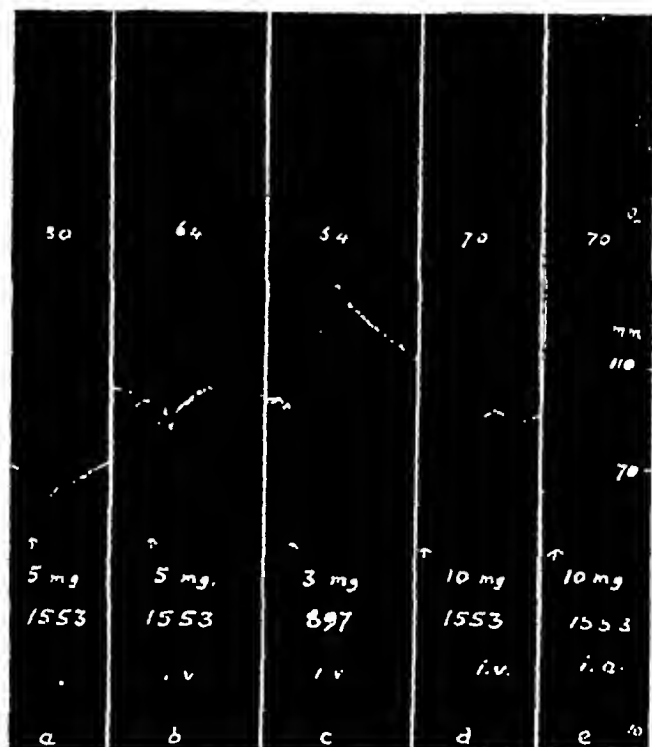


FIG 1.—Rabbit, urethane anaesthesia. Between *a* and *b* vagotomized and carotid sinus denervated. Upper record respiration, figures indicate rate per min. Lower record blood pressure.

In decerebrate and spinal cats a fall of blood pressure was produced which was, however, much less in the spinal preparation. In the latter preparation a marked increase in the pressor response to adrenaline was observed immediately following the intravenous injection of 1553 (Fig 2), in contrast to the decrease caused by the amidines (Wien, 1943, Dawes, 1945).

**Spleen Volume**—In decerebrate cats 1553 in doses of 1 to 2 mg injected intravenously or intra-arterially produced a marked increase in spleen volume (Fig 3) accompanied by a fall in blood pressure. There was little effect in the spinal cat, in which there was also little effect on the blood pressure.

*Heart*—On the isolated rabbit's heart (Langendorff's preparation), perfused with Ringer's solution, doses up to 1 mg caused slight stimulation in both rate and amplitude and some reduction of the effects of adrenaline. The effects were small and would be of little significance in the whole animal.

### Action on Smooth Muscle

On the isolated rabbit's intestine suspended in a bath of 50 c.c. of Tyrode's solution, doses up to 5 mg caused stimulation with increase in tonus, while larger doses produced relaxation and inhibition of the movements. The compound 897 had a much greater stimulant action, being about 25 times more active

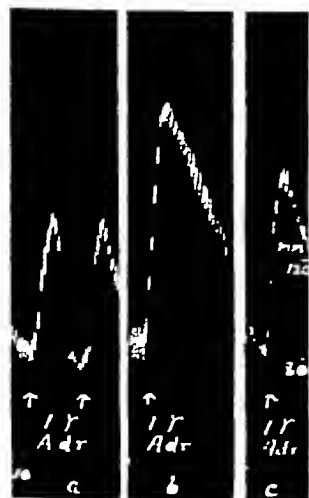


Fig. 2

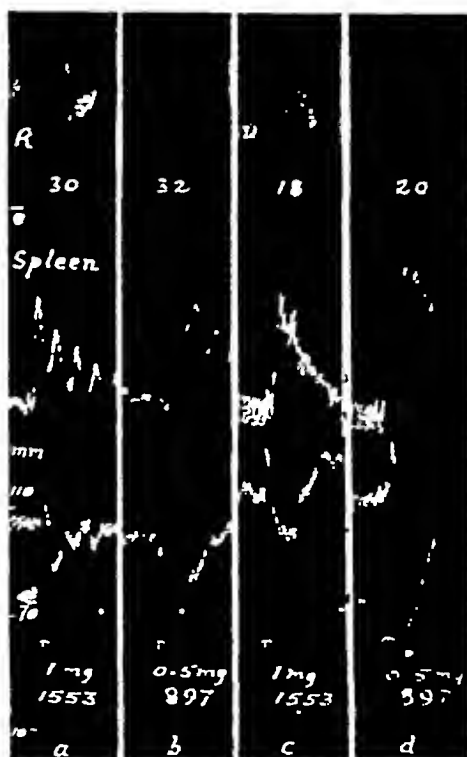


Fig. 3

FIG 2.—Cat, spinal. Blood pressure record. All injections intravenously. Between *a* and *b* 10 mg. 1553, which had only a slight depressor effect, but note that 1553 increased the response to adrenaline. At *c* the adrenaline response after 30 minutes was still raised.

FIG 3.—Cat, decerebrate. All injections intravenously. Between *b* and *c* vagotomized. Upper record respiration, figures indicate rate per min. Middle record spleen volume. Lower record blood pressure.

*Action on Blood Sugar and Blood Urea*

Six rabbits were used. In doses of 1 to 10 mg/kg given intravenously there was no significant effect on the blood sugar. In doses of 1 to 5 mg/kg there was little effect on the blood urea, but with 10 mg/kg an increase in the blood urea was usually observed. The effect was more pronounced if further injections were given later. The following figures illustrate this.

## EFFECT ON BLOOD UREA

Rabbits given (a) 2 daily subcutaneous injections of 10 mg/kg. of 1553, (b) the same injections repeated after 14 days.

Blood urea mg./100 c.c.	(a) (b)	44 63	68 102	80 101	122 248	84 —
Hours		0	5	24	48	120

The high blood urea levels were accompanied by proteuria, and at post mortem there was slight fatty degeneration and cloudy swelling in the tubules of the kidney.

*Action on the Liver*

Guinea pigs were used, since it has been found that they are more sensitive than other animals to the hepato-toxic action of the amidines in demonstrating fatty degeneration. The phenanthridinium compound 1553 was given intramuscularly in daily injections of 0.5, 1.0, 2.0 and 5.0 mg/kg for 5 days. The animals were killed some days after the end of treatment, and frozen sections of the liver were stained with sudan. On the lowest dose no pathological changes were observed, but with 1.0 mg/kg there was some fatty degeneration, and with 2 and 5 mg/kg the fatty degenerative changes were pronounced, resembling in this respect the toxic effects of the amidines.

*Action on Urinary Porphyrins*

Since Bell (1945) has found photosensitization effects in cattle, it was of interest to see whether this was associated with a porphyrinuria, but no increase in urinary porphyrins was found in rats even after 15 daily subcutaneous injections of 10 mg/kg.

## DISCUSSION

A consideration of the results obtained with the phenanthridinium compounds showed certain differences from those obtained by Browning and Calver (1943) and Calver (1945). They found that strain I (chronic infection) responded much better to treatment with 897 than strain II (acute infection), the C.D 50 figures being respectively 0.0005 and 0.005 mg/g—that is, a tenfold difference. They found also that treatment was much more effective at the acme stage, when parasites were abundant, than when few parasites were present. In the latter

case the above doses produced cures not in 50 per cent of the mice but in 0-6 per cent. With both strains they observed that if the acme stage was passed and a chronic stage ensued the trypanosomes were far more resistant, and the approximate C D 50 figures were increased to 0.005 and 0.025 mg/g respectively. We have not, however, observed these differences between the two strains in their response to treatment. The figures found in these experiments for the C D 50 for strains I and II were 0.0027 (limits 49-205 per cent,  $p=99$ ) and 0.002 mg/g (limits 54-185 per cent,  $p=99$ ). Treatment was given when trypanosomes were present to the extent of up to 20 per microscopic field. This infection may have been lighter than that employed by Browning and Calver, and may account for some difference in sensitivity, but it would not explain the lack of difference in response between the two strains. The passaging of the strains in different mice may also have some effect, although the characteristic virulence of each strain was maintained. Confirmation of these observations was provided by the results obtained with the other chronic strain, V, against which the phenanthridinium compound was even less effective and gave a C D.50 figure of 0.015 mg/g, (limits 50-203 per cent.,  $p=99$ ). We were unable therefore to find that 897 was more effective in chronic than in acute infections.

Comparison of the results obtained with the phenanthridinium compound 897 and the diamidine 991 showed that they had the same therapeutic indices except for the chronic strain V, against which 897 was less active. Our results are therefore in agreement with those of Fulton and Yorke (1943), who found 991 as active as 897, but differ from those of Calver (1945), who found it considerably less active.

An analysis of the results obtained with the series of substituted compounds tested revealed some interesting relationships between chemical structure and trypanocidal activity. Although *T. congolense* infections only are considered here, the compounds have been examined for activity against other trypanosomes, and it may be stated that nuclear substitution in the 2-position in 4,4'-diamidinostilbene by Me, OH, Cl, Br or I led to a marked increase in activity against *T. equiperdum* and *T. rhodesiense* infections. These observations will be published in detail in another paper.

Walls and Browning (1945) have already pointed out the specificity of the action of the phenanthridinium compounds, greatest activity was obtained with the 2-amino substituents. While they are highly active against *T. congolense* they have only slight activity against *T. brucei* and *T. equiperdum*. A certain type of chemical structure, different from that required for other trypanosomes, is obviously required for *T. congolense*. Conversely, whereas the nuclear substituted stilbamidine derivatives are highly active against *T. equiperdum* and *T. rhodesiense*, they show little activity against *T. congolense*.

The active amidine compounds, 991, 1005, and 1311, in the stilbamidine series have a methyl substituted grouping, but in the phenanthridine series 3-amino-9,10-dimethylphenanthridine and 3-amino-9-methylphenanthridine are inactive.

(Morgan and Walls, 1932, 1938) Substitution in the unsaturated linkage of the amidines was more effective than nuclear substitution, and the dimethyl was more active than the monomethyl derivative. One requirement, therefore, appeared to be the presence of a methyl grouping, although amino substituted amidines, 1015 and 1025, had some activity, and a more interesting compound, 1150 (a phenanthrene diamidine) had no methyl grouping. This compound displayed a fairly high order of activity, and had a therapeutic index of at least 16. The C.D.50 has not been evaluated definitely since insufficient material was available. It is only slightly active (therapeutic index=3) against *T. equiperdum*, thus showing specificity of action against *T. congolense*. The preparation of the compound has been described by Barber and Stickings (1945). It is also of interest since it lacks a quaternary nitrogen atom, which is apparently necessary for optimal activity in the salts of the phenanthridinium compounds (Morgan and Walls, 1938). The examination of an analogous compound in which the amidino substituents are replaced by amino substituents would determine the relative importance of the amidine groupings. It was found, however, that an analogous compound, 3,6-diamidinocarbazole, was inactive, in this compound the amidine groupings were retained but the phenanthrene was replaced by a carbazole nucleus.

The compounds 1325, 1324, 193 to 198 and 1355 are all phenanthridinium compounds and are substituted in the positions shown by Walls to confer optimal activity. The compounds 1325 and 1324 are 9- $\beta$ -pyridyl-phenanthridines (Petrov and Wragg, 1946), and it was found that the replacement of the 9-aminophenyl grouping by 9- $\beta$ -pyridyl led to a reduction in activity. The 3-amino derivative (1325) showed slight activity, while the 7-amino derivative (1324) was inactive. Both compounds were also inactive against *T. equiperdum*. With compounds 193 to 198 we have studied the effect of the introduction of an unsaturated linkage between phenyl at C 9 and the phenanthridine nucleus. These compounds have a structural resemblance to the trypanocidal styryl-quinoline derivatives of Browning, Cohen, Ellingsworth and Gulbrandsen (1929). They were found to be completely inactive with the exception of the 7-amino derivative (197), which was slightly active against *T. congolense* but inactive against *T. equiperdum*. The substitution of amidine for amino groups in the phenanthridinium compounds, as in 1355, led to a loss of trypanocidal activity. It is interesting to note that although the introduction of an unsaturated linkage led to a decrease in trypanocidal activity, it produced an increase in antibacterial activity. Similarly the substituted amidines in the diphenoxy series were highly active bactericides although they were of little interest as trypanocidal agents.

The activity of the phenanthridinium compound 1553 (Walls and Browning, 1945) was evaluated for comparative purposes against *T. congolense* strain III. By subcutaneous injection the E.D.50 was found to be 0.00005 mg/g, the C.D.50 0.004 mg/g, and the L.D.50 0.061 mg/g, giving a therapeutic index of 152. This is of course valid only for mice. In cattle Bell (1945) has found recently an index of only 6.

A brief examination of the pharmacological actions of the phenanthridinium compounds 1553 and 897 has shown a similarity in some respects to the properties of the amidines. They have a depressant action on the circulation and are toxic to the liver, the phenanthridinium compounds also have a depressant action on the respiratory centre. Of some interest was the finding that 1553 increases the pressor effects of adrenaline, whereas the amidines cause a decrease. Although photosensitization symptoms have been reported in cattle we have been unable to find any increased output of porphyrins in the urine of experimental animals. It is quite possible that there may be an accumulation of porphyrin pigments in the liver which is not accompanied by any increased porphyrinuria. The toxic effects in cattle, where the animals may die six weeks after injection, are indeed suggestive of hepatic and renal degenerative changes.

### SUMMARY

1 A comparison has been made of the activities of 7-amino-9-*p*-aminophenyl-10-methylphenanthridinium chloride (897) and 4,4'-diamidino- $\alpha,\beta$ -dimethylstilbene dihydrochloride (991) against several acute and chronic strains of *T. congolense*. The phenanthridinium compound was twice as toxic and active against the acute strains, the therapeutic indices being the same, but against one chronic strain it was less active.

2 An evaluation of 2,7-diamino-9-phenyl-10-methylphenanthridinium bromide (1553) showed that by subcutaneous injection in acute infections the C.D. 50 was 0.0004 mg/g, the L.D. 50 was 0.061 mg/g, and the therapeutic index 152.

3 A study of the correlation between chemical structure and *T. congolense* activity led to the following conclusions:

#### *Diamidine Compounds*

(a) The introduction of methyl groupings into the unsaturated stilbene linkage enhanced activity, nuclear substitution was not so effective.

(b) Nuclear substitution by various groupings in the 2-position did not increase activity, except for a slight effect with methyl, amino and acetamido derivatives, although it was found that the introduction of halogen markedly increased activity against *T. equiperdum* (results to be published). Alkyl substitution in the amidine grouping and substitution of a diphenylamine for the stilbene linkage were without effect.

(c) The comparatively high activity of phenanthrene-3,6-diamidine was of interest. An analogous compound, 3,6-diamidinocarbazole, was devoid of activity.

#### *Phenanthridinium Compounds*

(a) The replacement of amino by amidine groupings resulted in loss of activity.



(b) The substitution of the 9-(3'-aminophenyl) by the 9-(3'-pyridyl) group led to a reduction in activity

(c) The introduction of an unsaturated linkage (*p*-dimethylaminostyryl) between phenyl at C 9 and the phenanthridine nucleus also led to a reduction in activity

4 Some of the pharmacological properties of the phenanthridinium compound 1553 have been described. In general it has a depressant action on the circulation and respiratory centre, and in high doses a toxic action on the liver and kidneys

I am indebted to Mr G Newbery and Dr H. J. Barber for the compounds examined with the exception of Nos 193 to 198, which were prepared for the Therapeutic Research Corporation, London, and kindly supplied by Dr V. A. Petrow, Queen Mary College. I wish to acknowledge particularly the very valuable assistance given by Miss U. Pardoe, who considerably facilitated the investigation by her careful and painstaking microscopic examinations. Mr L. Joyner kindly performed the biochemical estimations. My thanks are due to the directors of May & Baker, Ltd., for permission to publish the results.

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# THE VARIATION IN THE TOXICITY OF PHOSGENE FOR SMALL ANIMALS WITH THE DURATION OF EXPOSURE

BY

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In so far as the toxic effects of a lethal inhalant depend upon the amount of material absorbed these effects must depend upon the dosage ( $Ct$ ) to which animals are exposed, where  $C$ =the concentration of the gas in mg per cubic metre and  $t$ =the time of exposure in minutes. It must also depend upon the rate of inhalation and the efficiency of absorption of the gas in the respiratory tract. The present experiments show quantitatively how the median lethal dose of phosgene expressed as the  $L(Ct)_{50}$  varies with the exposure time ( $t$ ). From the data it is possible to deduce the probable extent to which different species reduce their breathing rate in an irritant gas.

The expression for toxicity or lethal index ( $L$ ) advanced by Haber (cf. Prentiss, 1937) was

$$L = Ctv/G \quad (1)$$

where  $C$ =concentration of toxic substance,  $t$ =time of exposure,  $v$ =volume of air breathed in per minute, and  $G$ =body weight in Kg. In general, for any one species under the same conditions  $v/G$  is constant, so that for any one species  $Ct$  would be constant. Thus the lethal index, or the median lethal dose,  $L(Ct)_{50}$ , should be constant for any one species if the breathing rate is unchanged. American work on the toxicity of phosgene for dogs (Geiling, 1944, Prentiss, 1937) showed that the "lethal index" rose from 4,500 mg min/m<sup>3</sup> when  $t$  was 2 min to 12,000 mg min/m<sup>3</sup> when the exposure time was 75 min. This increase in the lethal dose with increase in exposure time can be corrected for on the assumption that the organism can eliminate a constant amount of the toxic agent. By introducing an elimination factor  $C_0$  which may be assumed to be equal to the just harmless concentration, the formula becomes

$$(C - C_0)t = L \quad (2)$$

which is more consistent for long exposure time. The American data on dogs

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referred to above fit in with the function  $(C - 100)t = L$  fairly well. This suggests that under the condition of the experiments a concentration of  $100 \text{ mg/m}^3$  would not be fatal except after exposure for an infinite time.

With short exposure times the lethal concentration of phosgene increases considerably as the time is reduced. With exposure times of less than one minute the  $L(Ct)50$  is very high probably because the animals breathe less and possibly because with the high concentrations required the phosgene is less completely absorbed by the tissues of the respiratory tract.

### EXPERIMENTAL PROCEDURE

The apparatus used is shown in Fig. 1. Air was sucked through the Bruhl jar containing the animals placed on a stand of wire mesh, and phosgene was introduced for a short period of predetermined duration, at a rate measured on the flowmeter. The contents of the chamber were sampled by sucking through absorption bubblers at a measured rate. The bubblers, which contained hexamine and caustic soda solution, were turned on prior to introduction of the phosgene and were allowed to run for half to one minute after the

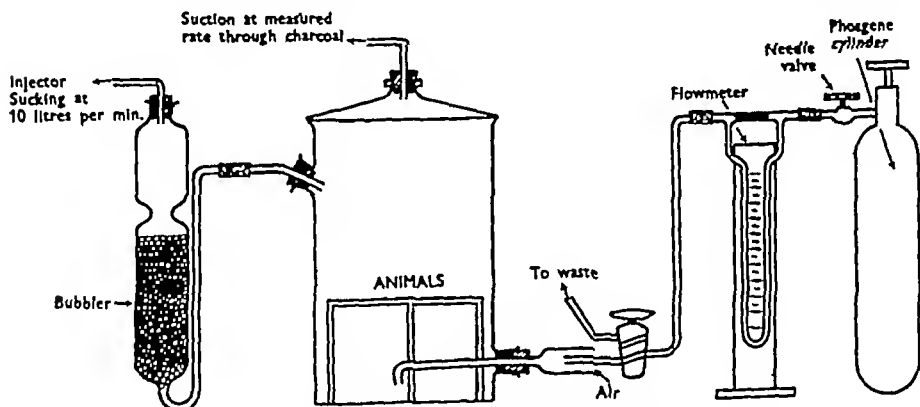


FIG 1—Apparatus used for short exposures of small animals to phosgene.

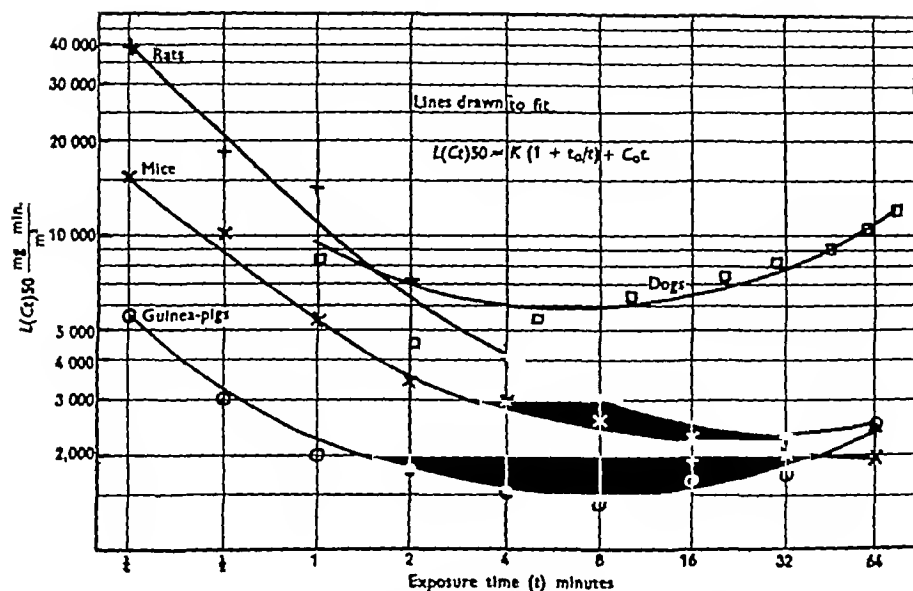
phosgene was turned off. The dosage ( $Ct$ ) to which the animals were subjected was calculated by dividing the weight of the phosgene collected (in mg) by the rate of sampling (expressed in  $\text{m}^3$  per min). Extra air was sucked through the jar and a charcoal filter and allowed to go to waste so that during exposures the total flow was  $30 \text{ l/min}$ . Exposures of 4 min or longer were carried out in a 20 litre chamber with the air passing through at  $200 \text{ l/min}$ .

### RESULTS

*Guinea-pigs*—The results obtained with guinea-pigs are given in Table I, and the relationship between the  $L(Ct)50$  and the time of exposure is plotted in Fig 2. The  $L(Ct)50$  is not constant, but is minimal at 8 min, and increases considerably with the shortening or lengthening of the exposure time. The corresponding curve for a constant concentration-time product on Fig 2 would be a straight horizontal line.

TABLE I  
MORTALITY OF GUINEA-PIGS EXPOSED TO PHOSGENE

Exposure time (t) min.	Dosage Ct mg. min./m. <sup>2</sup>	Mortality	L(Ct)50 (approx.) mg. min./m. <sup>2</sup>	Value for K, $\frac{L(Ct) 50 - C_0 t}{1 + t/t_0}$ t <sub>0</sub> = 0.8 min C <sub>0</sub> = 20 mg./m. <sup>2</sup>
½	7400 6400 4200 2700	6/6 2/3 1/6 0/6	5500	1310
½	7700 5100 4200 2350	6/6 4/6 2/3 3/6	3000	1130
1	8900 7500 4550 4000 3100 1700	3/3 3/3 5/6 3/3 8/9 2/6	2000	1190
2	8500 4400 1800 1575	6/6 6/6 3/6 2/7	1800	1260
4	2480 1915 1675 1425	4/6 9/10 8/10 4/10	1500	1180
8	2410 1760 1700 1620 1200	6/6 9/10 13/16 8/10 2/10	1400	1130
16	1900 1740 1660 1375	11/16 4/6 5/10 2/10	1660	1270
32	2275 1835 1800 1775 1700 1575	6/6 8/10 8/10 11/16 6/10 2/10	1700	1090
64	3480 3425 3125 2560	5/6 9/10 6/10 2/6	2900	1570

FIG 2—The  $L(Ct)50$  values for animals exposed to phosgene

*Rats and Mice*—The data in Tables II and III and Fig 2 show that the dosages necessary for 50 per cent mortality are high. The  $L(Ct)50$  for rats rises with shortening exposure time to a greater extent than with the other species examined.

*Dogs*—Mortality figures for dogs from American experiments are given in Table IV and are also indicated on Fig. 2. Dogs are less susceptible to phosgene than are the other species with long exposure times, but no figures are available of exposure times less than 1 min.

#### THE VARIATION IN $L(Ct)50$ WITH EXPOSURE TIME

The data summarized in Fig 2 show how enormously the  $L(Ct)50$  for most animals increases as the duration of exposure to phosgene is reduced. It is almost certain that this effect is due largely to reduction and modification of breathing in the presence of the gas, although it is possible that other factors may play a part, for example, the efficiency of absorption may be influenced by the concentration.

By inspection and empirical trial it was found that the data could best be fitted by an equation of the form

$$(C - C_0)t = K(1 + t_0/t) \quad (3)$$

where  $C$  is the  $LC50$  corresponding to a time  $t$ , and  $C_0$  and  $t_0$  are constants. The values of  $K$  for different times are seen in Tables I–IV, and mean values are

TABLE II  
MORTALITY OF RATS EXPOSED TO PHOSGENE

Exposure time (t) min	Dosage Cr mg min /m <sup>3</sup>	Mortality	L(Cr)50 (approx ) mg min /m <sup>3</sup>	Value for K, $\frac{L(Cr)50 - C_0 t}{1 + t_0/t}$ $t_0 = 4 \text{ min}$ $C_0 = 10 \text{ mg./m}^3$
$\frac{1}{2}$	41,500 29,000 20,000 16,000 10,000 6,200 3,200	7/12 3/12 5/16 2/8 1/4 0/4 0/4	39,000	2300
$\frac{1}{4}$	32,000 25,700 14,000 9,600 5,800	7/8 13/20 8/20 4/12 0/4	18,000	2000
1	56,000 32,000 27,000 21,700 12,500 5,700 2,900	8/8 3/4 7/8 10/12 3/12 0/4 0/4	14,000	2800
2	11,700 5,900 3,350 2,500	6/8 3/8 1/4 1/4	7,000	2320
4	6,100 5,200 2,900 2,475	10/12 3/4 3/10 1/10	4,000	1980
8	4,075 3,710 3,625 3,450 2,880 2,410	7/10 24/40 8/10 1/10 12/30 2/10	3,000	1950
16	2,930 1,900 1,740 1,400	14/20 3/10 0/10 1/10	2,400	1740
32	2,600 2,275 2,225 2,000 1,775 1,700	7/10 7/10 5/10 2/10 2/10 4/10	2,200	1670
64	3,500 3,480 2,975 2,875 2,560 2,500	10/10 10/10 21/30 2/10 2/10 4/10	2,800	2040

TABLE III  
MORTALITY OF MICE EXPOSED TO PHOSGENE

Exposure time (t) min	Dosage Ct mg. min /m. <sup>3</sup>	Mortality	$L(Ct) 50$ mg. min /m. <sup>3</sup>	Value for K, $\frac{L(Ct) 50}{1 + t_0/t}$ $t_0 = 1.6 \text{ min}$
$\frac{1}{2}$	19700 11500	20/20 7/20	15000	2020
$\frac{1}{2}$	17000 11200 7900	20/20 15/20 3/20	10000	2380
1	9500 9200 4200	20/20 19/20 5/20	5400	2080
2	5700 5000 3700 3450 3100 2600 1925	20/20 19/20 13/20 24/40 6/20 3/20 2/20	3400	1900
4	3525 2900 2475	17/20 8/20 1/10	3000	2140
8	3625 3450 2935 2410 1675	16/20 17/20 14/20 1/10 4/10	2500	2080
16	3450 3050 3025 2930 2300 2035 1950 1900 1740 1500 1400	14/20 20/20 10/20 20/20 12/20 8/20 8/20 9/10 2/10 2/10 0/10	2200	2000
32	2275 1800 1775 1700	8/10 5/20 3/10 7/20	2000	1900
64	3480 2560 2085 1775 1650	10/10 10/10 15/20 7/20 3/20	1900	1850

indicated in Table V The value of  $K$  is approximately equal to the minimum value of the  $L(Ct)50$  In discussing the significance of these constants it is convenient to consider short exposures and long exposures separately

TABLE IV

## MORTALITY OF DOGS EXPOSED TO PHOSGENE

(Data for 1 and 2 min exposures Geiling, 1944 Data for longer exposures Prentiss, 1937)

Exposure time ( $t$ ) min	$L(Ct)50$ mg. min / m <sup>3</sup>	Value for $K$ , $\frac{L(Ct)50 - C_0 t}{1 + t_0/t}$ $t_0 = 1$ min $C_0 = 100$ mg./m <sup>3</sup>
1	8400	4100
2	4500	2800
5	5500	4160
10	6500	5000
15	6900	5000
20	7400	5150
30	8100	4950
45	9000	4400
60	10,200	4130
75	12,000	4430

*Short Exposures*—In this case  $C_0$  is negligible compared with  $C$  and the expression (3) becomes approximately

$$Ct = K(1 + t_0/t) \quad (4)$$

Although the values of  $t_0$  used to fit these data were originally obtained by inspection and trial, Professor Gaddum has pointed out that they can also be determined by plotting  $Ct$  against  $1/t$ , this should give a straight line, and  $K$  is equal to the value of  $Ct$  when  $1/t$  is zero The constant  $t_0$  is the value of  $t$  when  $Ct = 2K$  (when  $C_0$  is neglected) and  $K$  is the value to which  $Ct$  approaches when  $t$  is infinite Theoretically it must be less than the minimum value of  $Ct$  Experimentally it was approximately equal to this quantity

The constant  $t_0$  is the time at which  $Ct$  is double this theoretical lower limit, it corresponds approximately to points on the curves shown in Fig 2 at a fixed height (log 2) above their minima If the increase of  $Ct$  with short times is entirely due to inhibition of the respiration, then the animals breathe, in a time  $t_0$ , half what they would have breathed under threshold conditions with prolonged exposures In many cases this is likely to be equal to half what they would have breathed in the absence of the gas

The observed values of  $t_0$  are given in Table V The values for rats and mice were larger than those for larger animals, this presumably indicates that rats and mice hold their breath longer than the other animals



TABLE V  
CONSTANTS OBTAINED FOR THE DIFFERENT SPECIES EXAMINED

Species	$t_0$ (min)	Minimal values of $L(Ct)50$ (mg. min /m <sup>3</sup> ) observed (K) theoretical		Value of $t$ at minimal value of $L(Ct)50$ (min)	$C_0$ safe concentration (mg./m <sup>3</sup> )
Rats	4	2200	2000	30	10
Mice	1.6	1900	2000	64	0
Guinea pigs	0.8	1400	1250	8	20
Dogs	1	4500	4500	5	100

The argument given above leads to the conclusion that  $K$  is an index of the toxicity of the gas, and that  $t_0$  is an index of the time for which the animals reduce their breathing to a mean value of half their normal rate

*Long Exposures*—For long exposures  $Ct$  was found to increase in some cases, presumably owing to detoxication of the phosgene. It has in fact been found that when  $C$  falls below a threshold concentration, death does not result, however long the exposure

When  $t$  is large the formula (3) given above becomes

$$(C - C_0)t = K \quad (5)$$

and the correction involving  $t_0$  can be neglected

#### DISCUSSION

With the data given, the expected  $L(Ct)50$  at any exposure time can be calculated from the equation

$$L(Ct)50 = K(1 + t_0/t) + C_0 t \quad (6)$$

using the constants given in Table V. The curves in Fig. 2 are drawn to this equation

No true minimum value of the  $L(Ct)50$  was found for mice, which indicates that  $C_0$  or the safe concentration is very low. Low concentrations which might be safe for other animals would probably cause death in mice on long exposure

The variations in toxicity with exposure time thus give an *indirect indication* of the extent to which animals hold their breath in clouds of phosgene. The times ( $t_0$ ) of reduced breathing may be measures of ability to hold the breath, or of reactions to the irritant gas or of combined effects of several factors

#### SUMMARY

The lethal dose expressed as the  $L(Ct)50$  of phosgene for small animals rises enormously as the exposure time is shortened. The extent of the rise varies with different species

The expression  $L(Ct)50(1 + t_0/t)$ , where  $t_0$  is a species constant, is reasonably constant for short exposures. Assuming that the increase of dosage ( $Ct$ ) with short time is entirely due to inhibition of respiration,  $t_0$  is an index of the time

for which the animals reduce their breathing by half, in the presence of the toxic gas. Thus the variation of toxicity with time gives an indication of the extent to which different species reduce breathing in the presence of an irritant gas.

We are indebted to Professor Gaddum for his interest in the treatment of the data and to the Chief Scientific Officer of the Ministry of Supply for permission to publish the results.

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## SYNTHETIC SUBSTITUTES FOR QUINIDINE

BY

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A preliminary account (Dawes, 1946) has already been given of a new method of measuring the activity of drugs as substitutes for quinidine, using the isolated auricles of the rabbit. It was found that many of the local anaesthetics and spasmolytics in common use had quinidine-like properties, and that some of them were intrinsically much more active than quinidine. This paper gives a fuller description of the method, and of the relation between chemical structure and quinidine-like activity.

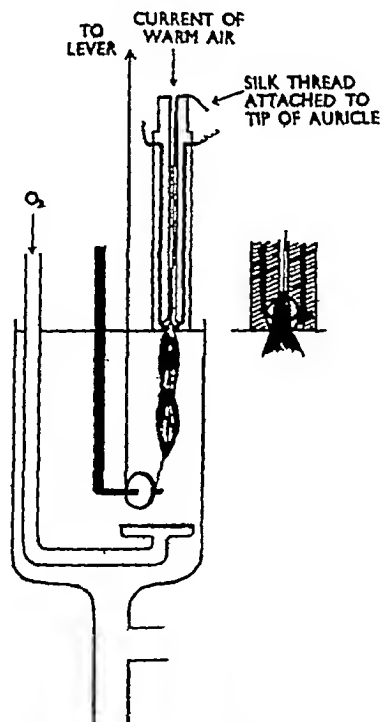


FIG. 1.—Preparation of rabbit auricles suspended in oxygenated Ringer-Locke at 29° C. The electrode holder is made of perspex, and the inset shows a closer view of its lower end.

## METHOD

Fig. 1 is a diagrammatic illustration of the preparation. The auricles are dissected from the heart of a rabbit and suspended in a bath of oxygenated Ringer-Locke at 29° C., at their upper end they are fixed in a pair of platinum electrodes just above the surface of the bath. The sharpened tips of the electrodes project into a tiny chamber at the bottom of a perspex rod. This chamber tapers at its lower end to form an oval opening, a silk thread is tied through the tip of one auricle, which is then drawn into the chamber so that the electrodes penetrate its substance, and so that the auricle itself completely seals the oval opening. At its upper end the chamber is continuous with a circular channel which runs through the perspex rod, a gentle current of warm air is blown down this channel at constant pressure, and serves the double purpose of oxygenating the tissue in the chamber and of preventing Ringer-Locke entering the chamber by capillary attraction, and so short-circuiting the electrodes. The object of this device is to ensure that, while the main part

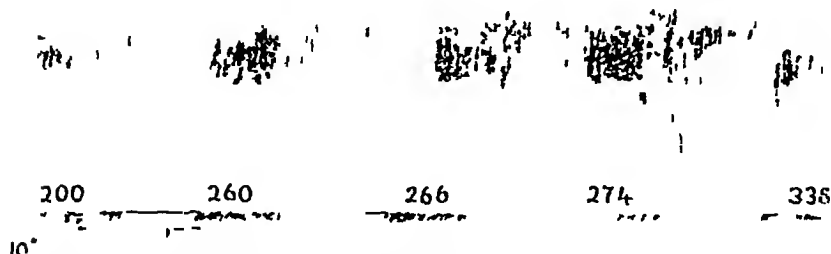


FIG 2—Record obtained from preparation shown in Fig 1. The upper signal-marker indicates the duration of stimulation by break induction shocks, the number of stimuli per minute is recorded above this. Up to 260/min the auricle responds to each stimulus, at 266 and 274/min it just fails to follow, at 338/min it adopts a 2:1 rhythm.

of the muscle is immersed in the bath, the electrodes are outside it. When methyl violet was added to the bath, the tip of the auricle drawn up into the electrode chamber was scarcely stained at all.

The contraction of the muscle is recorded by a lever writing on a smoked drum, and attached to the lower end of the auricles by a silk thread running round a pulley immersed in the bath. The auricles contract spontaneously (at a rate of 80–120 per minute), and they can also be stimulated by break-shocks from an inductor coil at any desired speed, using a Lewis rotary contact-breaker. The coil separation is adjusted so that the peak voltage in the secondary is about ten times that necessary to cause extrasystoles at the beginning of the experiment, this ensures that stimuli are so far above threshold that the notorious irregularity of induction shocks will not be of practical significance. As the rate of electrical stimulation is increased the auricle follows each stimulus up to a certain point (between 250 and 350 per minute) at which it begins to drop beats (because the interval between shocks is less than the absolute refractory period, cf Mines, 1913). It is easy to distinguish these dropped beats since the next auricular contraction is more powerful. Thus in the experiment shown in Fig. 2 the auricle followed each stimulus at 200 and 260 per minute, at 266 per minute it dropped a single beat, and at 274 per minute the response soon became very irregular, at 338 per minute it had adopted a 2:1 rhythm. In this instance the maximal rate at which the auricle could respond would be recorded as 260 per minute. The method is based upon the observation that quinidine reduces this maximal rate.

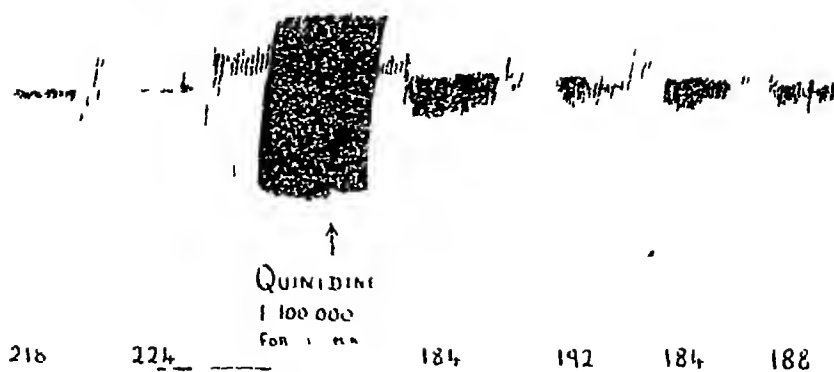


FIG 3—Preparation as in Fig 2. The maximal rate at which the auricle responds to electrical stimuli is 218/min, after quinidine 1 100,000 for 10 minutes this is reduced to 184/min. (The smoked drum was stopped for 9 minutes at the arrow, and was run at reduced speed immediately before and after it.)

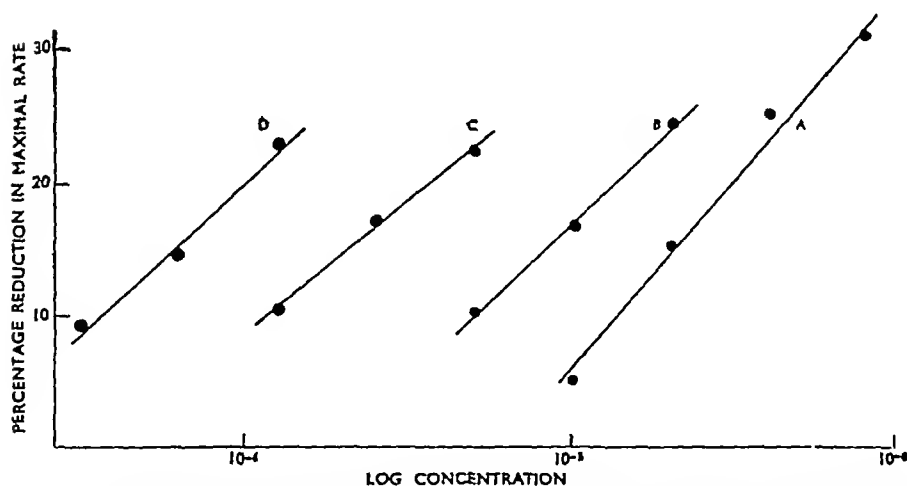


FIG 4—Ordinates: per cent reduction in maximal rate at which the auricle responds to electrical stimuli. Abscissae: concentration on a logarithmic scale. The points are the mean figures taken from (a) Quinine, 10 auricles, (b) Quinidine, 65 auricles, (c) compound 25, 10 auricles, and (d) Butethanol, 10 auricles.

The maximal rate is measured by applying stimuli at varying rates, each for 10–15 seconds (the maximal rate remains steady over long periods of time provided no drug is added to the bath). Quinidine is then added and the maximal rate measured again at the end of 10 minutes. Fig. 3 illustrates this procedure. Before the quinidine was added to the bath the maximal rate at which the auricles would follow electrical stimulation was 218 per minute, ten minutes after the addition of quinidine, the auricles followed stimuli

at 184 per minute, but not at 188 per minute, i.e., the maximal rate was reduced by 34 per minute or 15.6 per cent. Preliminary experiments with quinine and quinidine showed that the percentage reduction in the maximal rate bore a linear relationship to the logarithm of the concentration. As Fig. 4 shows, the slope of this line for various drugs is sufficiently parallel to allow their relative activity to be expressed in a single figure. Quinidine is used as the standard for comparison on each preparation.

The auricle returns to its initial maximum rate after the use of quinidine and closely related compounds rather slowly, some cumulation of effect inevitably occurs during the course of the assay, although an interval of 45-60 minutes is normally allowed between each determination. This cumulation does not interfere seriously with the assay, since the percentage reduction of the maximal rate caused by a given dose is, within wide limits, independent of the initial maximal rate. However, in order to reduce this source of error to a minimum it is expedient to use concentrations of quinidine (or of quinidine substitutes) which cause not less than 10 per cent and not more than 30 per cent reduction of the maximal rate.

Some idea of the error of the method may be obtained by a consideration of the standard deviations recorded in Table I for those compounds which were tested upon 5 or more auricles each.

## RESULTS

The relative activities of a number of substances are presented in Table I

TABLE I

Q=4 quinolyl    N=α-naphthyl    Ph=phenyl

Name or Number	Formula	Activity (Quinidine=1)	Number of Auricles used	Molar Weight	Activity per mol	LD 50 mg/kg. mice IP	Therapeutic Index
Quinidine	$  \begin{array}{c}  \text{CH} \\  \diagup \quad \diagdown \\  \text{CH}_2 \text{ CH}_2 \text{ CH CH CH}_2 \\  \diagdown \quad \diagup \\  \text{CH} \quad \text{CH}_2 \text{ CH}_2 \\    \\  \text{N}  \end{array}  $ 6-MeO Q CHO.H 2H <sub>2</sub> O	1.0	—	360	1.0	135	1.0
1	$  \begin{array}{c}  \text{CH} \\  \diagup \quad \diagdown \\  \text{CH}_2 \text{ CH}_2 \text{ CH} \cdot \text{CHOH CH}_2 \\  \diagdown \quad \diagup \\  \text{CH} \quad \text{CH}_2 \text{ CH}_2 \\    \\  \text{N}  \end{array}  $ 6-MeO Q CHO.H 2HBr	0.12	2	504	0.17	—	—
2	$  \begin{array}{c}  \text{CH} \\  \diagup \quad \diagdown \\  \text{CH}_2 \text{ CH}_2 \text{ CH} \cdot \text{CH}_2 \text{OH} \\  \diagdown \quad \diagup \\  \text{CH} \quad \text{CH}_2 \text{ CH}_2 \\    \\  \text{N}  \end{array}  $ 6-MeO Q CHO.H 2HBr	0.04	1	490	0.06	—	—

TABLE I (continued)  
 Q=4-quinolyl    N=a naphthyl    Ph=phenyl

Name or Number	Formula	Activity (Quinidine=1)	Number of Auricles used	Molar Weight	Activity per mol	LD50 mg./kg. mice IP	Therapeutic Index
Niquidine	$  \begin{array}{c}  \text{CH CH CH}_2 \\    \\  \text{CH} \\  / \quad \backslash \\  \text{CH}_2 \quad \text{CH}_2 \\    \quad   \\  \text{6-MeO Q CHO H} \text{CH} \quad \text{CH}_2 \\    \quad   \\  \text{NH} \quad \text{CH}_2  \end{array}  $ 2HCl	0.35	2	371	0.36	—	—
3	$  \begin{array}{c}  \text{CH}_2 \\  / \quad \backslash \\  \text{CH}_2 \quad \text{CH}_2 \\    \quad   \\  \text{6-MeO Q CHO H} \text{CH} \quad \text{CH}_2 \\    \quad   \\  \text{NH} \quad \text{CH}_2  \end{array}  $ HCl	0.14	2	308	0.12	—	—
4	$  \begin{array}{c}  \text{CH}_2 \quad \text{CH}_2 \\  / \quad \backslash \quad / \quad \backslash \\  \text{CH}_2 \quad \text{CH} \quad \text{CH}_2 \quad \text{CH}_2 \\    \quad   \quad   \quad   \\  \text{6-MeO Q CHO H} \text{CH}_2 \text{N} \quad \text{CH} \quad \text{CH}_2 \\    \quad   \quad   \quad   \\  \text{CH}_2 \quad \text{CH}_2 \quad \text{CH}_2 \quad \text{CH}_2  \end{array}  $ 2HCl	0.82	2	412	0.94	190	1.2
5	$  \begin{array}{c}  \text{CH}_2 \\  / \quad \backslash \\  \text{CH}_2 \quad \text{CH C}_6\text{H}_5 \\    \quad   \\  \text{6-MeO Q CHO H} \text{CH}_2 \text{N} \quad \text{CH}_2 \\    \quad   \\  \text{CH}_2 \quad \text{CH}_2  \end{array}  $ 2HCl	1.05	2	400	1.16	—	—
6	$  \begin{array}{c}  \text{CH}_2 \text{---} \text{CH}_2 \\  / \quad \backslash \\  \text{CH}_2 \text{---} \text{O} \\    \quad   \\  \text{6-MeO Q CHO H} \text{CH}_2 \text{N} \quad \text{CH}_2 \text{---} \text{CH}_2  \end{array}  $ 2HCl	0.10	2	360	0.10	—	—
7	$  \begin{array}{c}  \text{CH}_2 \text{---} \text{CH}_2 \quad \text{CH}_2 \text{---} \text{CH}_2 \\  / \quad \backslash \quad / \quad \backslash \\  \text{CH}_2 \text{---} \text{CH} \quad \text{CH} \quad \text{NH} \\    \quad   \quad   \quad   \\  \text{6-MeO Q CHO H} \text{CH}_2 \text{N} \quad \text{CH}_2 \text{---} \text{CH}_2 \quad \text{CH}_2 \text{---} \text{CH}_2  \end{array}  $ 3HCl	0.10	1	478	0.13	—	—

TABLE I (continued)  
 Q=4-quinolyl    N=α-naphthyl    Ph=phenyl

Name or Number	Formula	Activity (Quinidine=1)	Number of Auricles used	Molar Weight	Activity per mol	LD50 mg/kg. mice IP	Therapeutic Index
8	$  \begin{array}{c}  \text{C}_2\text{H}_5 \\    \\  \text{CH}-\text{CH}_2 \\    \quad \quad   \\  \text{6-MeO Q CHO H} \cdot \text{CH}_2 \cdot \text{CH} \quad \quad \text{NH} \\    \quad \quad   \\  \text{CH}_2-\text{CH}_2 \\    \\  \text{HNO}_3  \end{array}  $	0.96	2	391	1.05	150	1.1
9	$  \begin{array}{c}  \text{C}_2\text{H}_5 \\    \\  \text{6-MeO Q CHO H} \cdot \text{CH} \cdot \text{NH}_2  \end{array}  $ 2HBr	0.35	3	422	0.41	—	—
10	$  \begin{array}{c}  \text{6-MeO Q CHO H} \cdot \text{CH}_2 \cdot \text{N}(\text{C}_2\text{H}_5)_2  \end{array}  $ 2HCl	0.46	2	347	0.46	—	—
11	$  \begin{array}{c}  \text{6-MeO Q CHO H} \cdot \text{CH}_2 \cdot \text{N}(\text{C}_4\text{H}_9)_2  \end{array}  $ 2HCl	1.9	3	403	2.14	175	2.5
12	$  \begin{array}{c}  \text{6-MeO Q CHO H} \cdot \text{CH}_2 \cdot \text{N}(\text{C}_3\text{H}_7)_2  \end{array}  $ 2HCl	0.49	2	431	0.59	—	—
13	$  \begin{array}{c}  \text{Q CHO H} \cdot \text{CH}_2 \cdot \text{N}(\text{C}_2\text{H}_5)_2  \end{array}  $ 2HCl	0.43	1	317	0.39	—	—
14	$  \begin{array}{c}  \text{CH}_2-\text{CH}_2 \\    \quad \quad   \\  \text{6-MeO Q CHO H} \cdot \text{CH}_2 \cdot \text{N} \quad \quad \text{CH}_2 \\    \quad \quad   \\  \text{CH}_2-\text{CH}_2  \end{array}  $ HCl	0.55	3	322	0.50	260	1.0
15	$  \begin{array}{c}  \text{CH}_2-\text{CH}_2 \\    \quad \quad   \\  \text{7-MeO N CHO H} \cdot \text{CH}_2 \cdot \text{N} \quad \quad \text{CH}_2 \\    \quad \quad   \\  \text{CH}_2-\text{CH}_2  \end{array}  $ HCl	3.4	4	321	3.0	(175)	4.4
16	$  \begin{array}{c}  \text{CH}_2-\text{CH}_2 \\    \quad \quad   \\  \text{N CHO H} \cdot \text{CH}_2 \cdot \text{N} \quad \quad \text{CH}_2 \\    \quad \quad   \\  \text{CH}_2-\text{CH}_2  \end{array}  $ HCl	2.8 ± 0.11	5	291	2.3	200 (250)	4.1
17	$  \begin{array}{c}  \text{CH}_2-\text{CH}_2 \\    \quad \quad   \\  \text{Ph} \text{---} \text{CHO H} \cdot \text{CH}_2 \cdot \text{N} \quad \quad \text{CH}_2 \\    \quad \quad   \\  \text{CH}_2-\text{CH}_2  \end{array}  $ HCl	2.4	4	317	2.1	170 (175)	3.0
18	$  \begin{array}{c}  \text{CH}_2-\text{CH}_2 \\    \quad \quad   \\  \text{Ph} \cdot \text{CHO H} \cdot \text{CH}_2 \cdot \text{N} \quad \quad \text{CH}_2 \\    \quad \quad   \\  \text{CH}_2-\text{CH}_2  \end{array}  $ HCl	1.4	2	241	0.94	(175)	1.8



TABLE I (continued)

Q=4-quinolyl N= $\alpha$  naphthyl Ph=phenyl

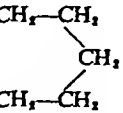
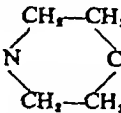
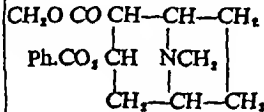
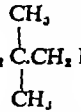
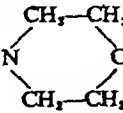
Name or Number	Formula	Activity (Quinidine=1)	Number of Auricles used	Molar Weight	Activity per mol	LD50 mg./kg mice IP	Therapeutic Index
19	$n\text{-C}_{11}\text{H}_{23}$ CHOH CH <sub>2</sub> N  HCl	0.18	3	319	0.15	(400)	0.53
20	Ph CO CH <sub>2</sub> N(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub> HBr	0.32	2	272	0.24	—	—
21	Ph CO CH <sub>2</sub> N  HBr	0.90	2	284	0.71	65	0.43
Cocaine	 HCl	6.2	5	325	5.6	—	—
Procaine	$p\text{-NH}_2$ Ph CO <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> N(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub> HCl	0.8	3	272	0.6	180 (250)	1.1
Butethanol	$p\text{-C}_6\text{H}_4\text{NH}$ Ph CO <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> N(CH <sub>3</sub> ) <sub>2</sub> HCl	13.8 $\pm$ 4.6	10	300	11.5	70	6.4
Butyn	$p\text{-NH}_2$ Ph CO <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> N(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub> H <sub>2</sub> SO <sub>4</sub>	5.5	4	355	5.3	80	3.3
Syntropan	Ph CH CO <sub>2</sub> CH <sub>2</sub>  N(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub> H <sub>3</sub> PO	1.3	2	405	1.5	—	—
Trasentin	Ph <sub>2</sub> CH CO <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> N(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub> HCl	0.63	2	347	0.59	—	—
22	Ph <sub>2</sub> C(OH) CO <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> N(CH <sub>3</sub> ) <sub>2</sub> HCl	2.1	2	335	2.0	—	—
23	Ph <sub>2</sub> C(OH) CO <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> N(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub> HCl	3.0	4	363	3.0	160	3.5
24	Ph <sub>2</sub> C(OH) CO <sub>2</sub> CH <sub>2</sub> N(CHMe <sub>2</sub> ) <sub>2</sub> HCl	6.8 $\pm$ 1.1	6	391	7.4	155	7.8
25	Ph <sub>2</sub> C(OH) CO <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> N  HCl	5.4 $\pm$ 1.9	10	375	5.6	150	6.0

TABLE I (continued)  
 Q=4-quinolyl N= $\alpha$ -naphthyl Ph=phenyl

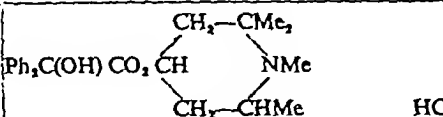
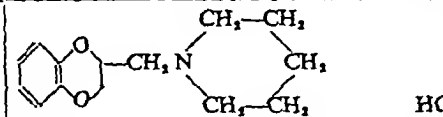
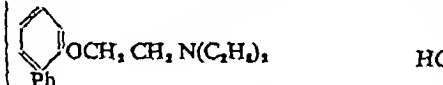
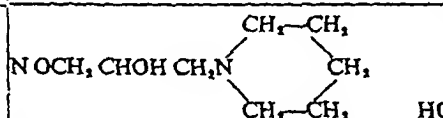
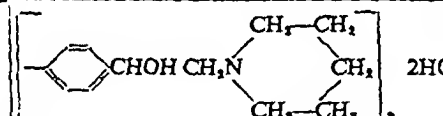
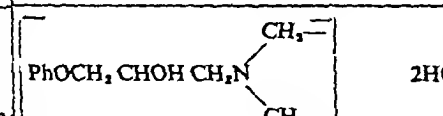
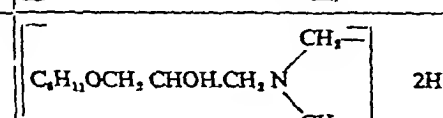
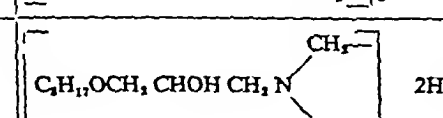
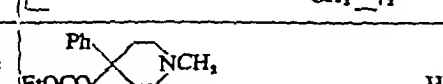
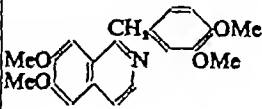
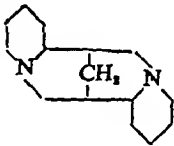
Name or Number	Formula	Activity (Quinidine=1)	Number of Auricles used	Molar Weight	Activity per mol	LD50 mg./kg mice IP	Therapeutic Index
26	 HCl	4.6	4	403	5.1	75	2.6
F933	 HCl	3.2	4	269	2.4	180	4.3
F1262	 HCl	4.7 $\pm$ 0.67	5	305	3.9	125	4.4
27	 HCl	4.4 $\pm$ 1.4	5	321	4.0	150	4.8
28	 2HCl	0.17	1	481	0.23	—	—
lower m.p. 29 higher m.p.	 2HCl	3.2 2.1	4 2	459 459	4.4 2.9	140 —	4.1 —
30	 2HCl	0.63	2	471	0.82	—	—
31	 2HCl	<0.10	1	531	—	—	—
Pethidine	 HCl	0.83	2	283	0.65	—	—

TABLE I (continued)

Q=4-quinolyl N=α-naphthyl Ph=phenyl

Name or Number	Formula	Activity (Quinidine=1)	Number of Auricles used	Molar Weight	Activity per mol	LD50 mg./kg. mice IP	Therapeutic Index
Phenacaine	$\begin{array}{c} p\text{-EtO Ph NH} \\ p\text{-EtO Ph N} \end{array} \text{C} \cdot \text{CH}_3$	HCl 4.5	4	334	4.2	80	2.7
Papaverine		HCl 0.50	3	375	0.52	—	—
Sparteine		H <sub>2</sub> SO <sub>4</sub> 0.34	2	422	0.40	—	—

The toxicity figures in parentheses are taken from MacIntosh and Work (1941) for substances in short supply. Their figures for compounds 16 and 17 and procaine afford a comparison with results obtained in this laboratory.

The base of compound 29 exists in two, probably stereoisomeric, forms (α) melts to an opaque liquid at 89° which becomes clear at 102° C., hydrochloride, m.p. 238° efferv., (β) melts to an opaque liquid at 136° which becomes clear at 144° C., hydrochloride, m.p. 247° efferv. (personal communication from Dr. H. R. Ing.)

The third column records their activity in terms of quinidine=1.0, weight for weight. In all these experiments quinidine (Howards) was weighed as base, dissolved in dilute hydrochloric acid and neutralized. All other substances were weighed as crystalline salts. The fourth column records the number of auricles on which the substance has been assayed against quinidine. The sixth column records the activity per molecule, and the seventh column the LD<sub>50</sub> on intraperitoneal injection into mice. The index of therapeutic efficiency in the eighth column is calculated as

$$\frac{\text{Activity} \times \text{LD}_{50} \text{ of new compound}}{\text{Activity} \times \text{LD}_{50} \text{ of quinidine}}$$

The choice of substances tested may require some explanation. The investigation began with a series of compounds (Nos. 1–19 and 28) which Drs. Harold King and T. S. Work had made in a search for new anti-malarials. Later the work was extended to include local anaesthetics and the benzilic ester series.

(Nos 22-26), partly because of previous work on the prevention of experimental fibrillation by local anaesthetics and partly because of the structural resemblance between alkamine esters of aromatic acids and the anti-malarials prepared by King and Work. Finally some other compounds which were reputed to prevent experimental fibrillation (such as F 993, F 1262, papaverine and sparteine) were included in order to cover as wide a field as possible. The omission of some compounds was unavoidable (for references see Bijlsma and van Dongen, 1939, Jack, 1942-3, Deulofen *et al*, 1945), and as most of the synthetic drugs in Table I were made with very different purposes in mind, there are numerous gaps in homologous series which it would have been interesting to have filled, if the compounds had been at my disposal.

Inspection of Table I shows that quinidine-like activity is displayed by a very large range of compounds. The majority of these contain an aromatic group joined to a basic group by a carbinol, keto, ester or ether linkage. Atropine also satisfies these conditions (cf. syntropan, which is also an ester of tropic acid) and in a concentration greater than 1/25,000 has a quinidine-like action upon the auricle. This concentration is of course many times greater than that required to antagonize the action of acetylcholine. Pethidine, phenacaine and papaverine possess both aromatic and basic groups and have a quinidine-like action, similarly the rosaniline dyes, methyl violet and ethyl violet, which also possess these two groups, have a quinidine-like action in very high concentrations (1/10,000 or more).

Compounds 22-26, tertiary amino-alkyl esters of benzoic acid, were originally made during a search for atropine substitutes (Ing, Dawes and Wayda, 1945). Several analogous quaternary salts were available, three of which were tested upon the rabbit auricle. These were benzilylcholine chloride and benzilyloxyethyl-dimethylethylammonium chloride (Lachesine, until recently known as E3), the metho- and etho-chlorides respectively of compound 22, and benzilyloxyethyl-diethylmethylammonium chloride, the metho-chloride of compound 23. These three quaternary salts were quite inactive when tested on the auricle in concentrations of up to 1/5,000.

Of the forty-four compounds in Table I twenty have a therapeutic efficiency index of 1.0 or more (i.e., equal to or greater than quinidine). Since intraperitoneal toxicity figures conceal such factors as rates of absorption and excretion, intravenous toxicity tests were also made on the seven compounds which had an index greater than 4.0. The results are shown in Table II, arranged in order of ascending therapeutic efficiency as judged by the intraperitoneal tests. The two benzoic ester derivatives (24 and 25) are outstanding in that they are still nearly three times as good as quinidine, even when injected intravenously. No. 25 has an LD<sub>50</sub> on oral administration to mice of 440 mg./kg., compared with 630 mg./kg. for quinidine, this gives it an oral therapeutic efficiency index of 3.7. It is suggested that this compound might be worth clinical trial as a substitute for quinidine in auricular fibrillation.

TABLE II

Name	Activity	LD50 Mice i p mg./kg.	Therapeutic Index	LD50 Mice i v mg./kg.	Therapeutic Index
Quinidine	1.0	135	1.0	65	1.0
No 16	2.8	200	4.1	35	1.5
F 933	3.2	180	4.3	35	1.7
F 1262	4.7	125	4.4	27	2.0
No 27	4.4	150	4.9	25	1.7
No 25	5.4	150	6.0	40	3.3
Butethanol	13.8	70	6.4	7.5	1.6
No 24	6.8	155	7.8	28	2.9

### Sympathomimetic Amines

Compound No 18 ( $\beta$ -phenyl  $\beta$ -hydroxyethylpiperidine) has a close structural resemblance to many sympathomimetic amines. MacIntosh and Work (1941) found that it had a diphasic effect on blood pressure and pulse rate, occasionally producing a purely pressor and accelerator response. Like cocaine, procaine (MacGregor, 1939, b), butyn and stovaine (Tripod, 1940), compound No 18 (as well as Nos 15, 16, 17, 19 and 28) potentiated the pressor action of small doses of adrenaline. When tested on the rabbit auricle it was found to be a little more active than quinidine and consequently a few sympathomimetic amines were tested on this preparation.

Adrenaline in concentrations of from 1:1,000,000 to 1:25,000 caused an increase in the maximal rate at which the auricle would respond to electrical stimuli. This is yet another instance of the stimulant action of adrenaline upon the heart.

The depressant action of large concentrations of ephedrine is well known (Chen and Schmidt, 1930). In one out of four auricles ephedrine in a concentration of 1:100,000 caused an increase in the maximal rate, in the other three experiments and in higher concentrations it caused a decrease (with a mean figure of 0.30 of the activity of quinidine). This quinidine-like action was accompanied by an increase in the spontaneous rate at which the auricles beat with low concentrations, but with higher concentrations (1:25,000 or more) there was a decrease both in spontaneous rate and amplitude. Methedrine and amphetamine had about the same quinidine-like activity as ephedrine upon the auricle,  $\beta$ -phenylethylamine was only half as active.

We may therefore conclude that, while many local anaesthetics which may in some respects be regarded as sympathomimetic possess a quinidine-like action

upon the auricle, yet other drugs which are usually thought of as predominantly sympathomimetic may in higher concentrations also act like quinidine. In this respect they are the very reverse of sympathomimetic.

#### *Action of Quinidine Substitutes on Pacemaker and Amplitude of Contraction*

For a given reduction in the maximal rate, butethanol caused a notably smaller decrease in the rate at which the auricles beat spontaneously than did quinidine. In ten experiments quinidine 1:100,000 caused a mean reduction in the maximal rate of 16.3 per cent and in the spontaneous rate of 15.2 per cent, butethanol 1:1,600,000 caused a mean reduction in the maximal rate of 14.6 per cent and in the spontaneous rate of only 3.0 per cent. None of the other quinidine substitutes with a high therapeutic efficiency index showed a similar difference. Similarly, while adrenaline increased both the spontaneous rate and the maximal rate of the auricles, and quinidine decreased both, acetylcholine in concentrations which almost stopped the auricle increased the maximal rate. On the other hand, ephedrine, and in some experiments cocaine, in low concentrations accelerated the spontaneous rhythm but decreased the maximal rate, while in high concentrations they decreased both. The action of these drugs upon the pulse-rate cannot therefore be relied upon as an indication of their effect upon the heart muscle.

The majority of the quinidine-substitutes listed in Table I cause a considerable decrease in the maximal rate of the auricles before they affect the amplitude of each contraction. Quinidine usually causes a 20 per cent decrease in the maximal rate before the amplitude is much reduced (see Fig. 3). Since it is inexpedient to use concentrations of a drug which cause more than a 30 per cent reduction in the maximal rate during the assay, it is difficult to judge accurately the relative effect upon the amplitude. The majority are certainly no more depressant (relatively) than is quinidine. No. 31 is a striking exception, for it caused a very large depression in a concentration of 1:10,000 without affecting the maximal rate. Pentobarbitone (nembutal) behaved in the same way. These differences between the relative activity of drugs upon the maximal rate of the auricle, the pacemaker, and the amplitude of each contraction do not lend support to the view that these drugs are 'general tissue poisons'. On the contrary, the property required in the ideal quinidine-substitute, which some of these substances go a certain way towards fulfilling, is that it should act principally upon the heart muscle, like digitalis; therefore, it should cause death from heart failure by an extension of its therapeutic action. This is the best possible insurance against untoward reactions, e.g., on the central nervous system. It is interesting in this connection to observe that compound 25, which was considered the most suitable for clinical trial on account of its high therapeutic efficiency index (low relative toxicity), has one of the lowest local anaesthetic activity to quinidine-like activity ratios (Fig. 5).

### Local Anaesthetic Activity

The local anaesthetic activity of these compounds has already been referred to. Quinine itself is reputed to possess mild local anaesthetic properties. MacIntosh and Work (1941) demonstrated the local anaesthetic activity of compounds 15, 16, 17, 18, 19 and 28. Gilman and his collaborators (1942) demonstrated the local anaesthetic activity of trasentin and compound 23. In this laboratory compounds 20 and 21 were found to have transient local anaesthetic activity on intradermal injection into the guinea-pig, compounds 23, 24, 25, 26, pethidine and F1262 were as active as, or more active than, procaine. With cocaine, procaine, butethanol, butyn and phenacaine this brings up to twenty-one the number of compounds in Table I which possess considerable

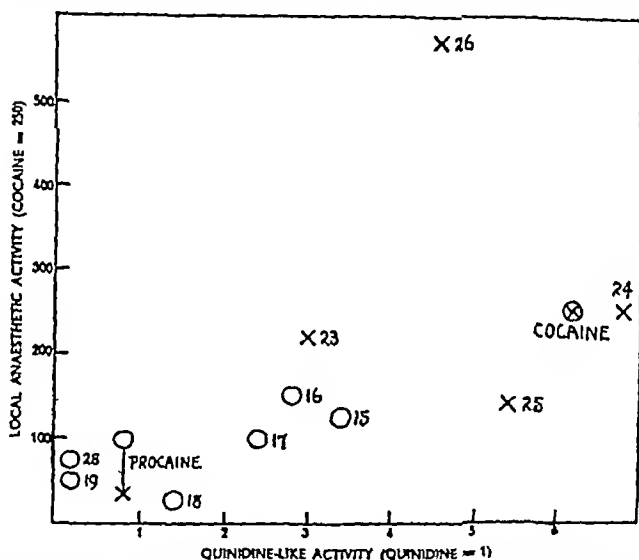


FIG 5—Ordinates Local anaesthetic activity (cocaine=250). Abscissae Quinidine-like activity (quinidine=1). Numbers refer to compounds in Table I. The data for local anaesthetic activity are taken from MacIntosh and Work (circles) and from Wajda (crosses).

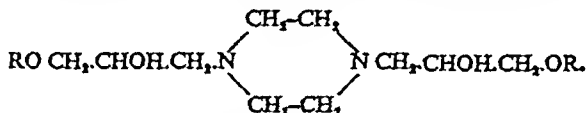
local anaesthetic activity. In addition there is nupercaine, the latter is difficult to assay upon the auricle since it causes a profound and prolonged depression of amplitude, as well as a reduction in the maximal rate at which the auricle will respond. Papaverine (Pal, 1914, Macht, Johnson and Bollinger 1916, Reynolds, 1940) has also been shown to possess feeble local anaesthetic properties.

Saligenin (*o*-hydroxybenzylalcohol) is a moderately powerful local anaesthetic of very different structure (Hirschfelder, Lundholm and Norrgard, 1920). It possesses no basic group, and it has no action upon the auricle even in concentrations of 1/5,000. Quinidine-like activity is not therefore invariably associated with local anaesthetic activity, it is possible that saligenin produces

its local anaesthetic action in a different way from the local anaesthetics in common use, which are mostly dialkylaminoalkyl esters of aromatic acids.

Although among the compounds in Table I local anaesthetic activity does not run parallel with quinidine-like activity, there is a fair measure of agreement. This point is illustrated in Fig 5, in which the quinidine-like activity estimated upon the rabbit auricle is plotted against the local anaesthetic activity estimated by intradermal injection into guinea-pigs of six piperidino-methyl carbamol derivatives (MacIntosh and Work, 1941) and four tertiary amino-alkyl esters of benzoic acid (Wajda 1946). Similarly, if the familiar local anaesthetics in Table I are put in order of quinidine-like activity (butethanol, cocaine, phenacaine, butyn and procaine), that also is the order of their activity in infiltration anaesthesia, so far as can be judged by a study of the relevant literature.

The relative local anaesthetic activity estimated upon the guinea-pig's cornea may show a 100-fold difference from that estimated upon the guinea-pig's skin (MacIntosh and Work, 1941). This is probably due to the introduction of an additional factor into the assay, viz., the rate of penetration of the cornea by the drug. Quinidine-like activity as estimated on the rabbit auricle should therefore be compared with *intrinsic* local anaesthetic activity, estimated by applying the drug as directly as possible to the nerve. For instance, Fourneau and Samdahl (1930) examined a series of piperazine derivatives of the type



When R was  $\text{C}_6\text{H}_{13}$  or  $\text{C}_7\text{H}_{15}$ , they had respectively 8 and 22 times the activity of cocaine upon the rabbit's cornea. In compound 31 R is  $\text{C}_8\text{H}_{17}$ , yet it fails to reduce the maximal rate of the rabbit's auricle in a concentration of 1/10,000, and when tested for local anaesthetic properties by intradermal injection into the guinea-pig it was found to have less than half of the activity of procaine.

### Spasmolytic Activity

Some of the compounds in Table I are well recognized as spasmolytics, e.g., syntropan, trasentin, pethidine and papaverine. Others undoubtedly possess the property of causing relaxation in isolated strips of intestine, though this has been described as a sympathomimetic action and may be preceded in low concentrations by a period of increased tone and amplitude, e.g., cocaine and procaine, (Roth, 1917, Macgregor, 1939, b), butyn and nupercaine (Tripod, 1940). Bovet, Fourneau, Tréfouël and Strickler (1939) found that F1262 had a spasmolytic action in the dog under chloralose, and antagonized the contraction produced by acetylcholine and barium chloride *in vitro*.

Quinidine and procaine (in a concentration of 1/100,000 to 1/25,000) also cause a reduction of tone in the isolated rabbit duodenum, suspended in oxygenated Ringer-Locke at 37°C. Both quinidine and procaine greatly reduce





The fact that quinidine and procaine reduced the action of acetylcholine upon the isolated intestine suggested that they might do so upon the heart. Starr (1936) has shown that quinidine diminishes or abolishes the ability of acetyl- $\beta$ -methylcholine and of acetylcholine to slow the heart rate in anaesthetized cats and in isolated cats' and rabbits' hearts. His observation regarding acetylcholine has been confirmed in the isolated rabbit auricle. Not only quinidine, but also procaine (Fig. 7) reduce the ability of acetylcholine to slow the rate and depress the amplitude of contraction of the isolated rabbit's auricle.

### DISCUSSION

The method described in this paper for measuring quinidine-like activity enables an estimate to be made of the reduction in the maximal rate at which isolated rabbit auricles will respond to electrical stimulation. This measurement was adopted as an index of quinidine-like activity because it was relatively simple and because a direct comparison could be made of two or more substances upon the same piece of tissue. Quinidine is believed to stop auricular fibrillation because it prolongs the refractory period. Lewis (1922) emphasized the fact that quinidine not only prolongs the refractory period, but also slows the conduction of excitation, a change which would act in the opposite direction and tend to perpetuate circus movement. Strictly speaking, therefore, an ideal method of testing quinidine substitutes should take into account the action of a drug on conduction rate as well as on refractory period. The method described, which involves the measurement of the maximal rate, depends principally upon the refractory period. However, a number of substances other than quinidine, which reduce the maximal rate of the rabbit auricle have already been shown to prevent or stop auricular or ventricular fibrillation induced by various methods in experimental animals. These include procaine (for references see Dawes, 1946), cocaine (Hermann and Jourdan, 1931), butethanol (under the name pantocaine, Hirschfelder and Tamcales, 1942), F1262 (Bovet, Fourneau, Tréfouel and Strickler, 1939), F933 (Shen, 1939, van Dongen, 1939), papaverine (Lindner and Katz, 1941, Elek and Katz, 1942, Wégria and Nickerson, 1942), and sparteine (Crawford 1926). It may therefore be reasonably supposed that drugs which are intrinsically more active than quinidine upon the auricle will stop auricular fibrillation in human beings, provided that a sufficiently high concentration can be maintained in the blood-stream over an adequate period of time without untoward reactions. (There is for instance, evidence that the protection afforded by procaine against cyclopropane-adrenaline ventricular tachycardia is more transient than that of quinidine, Meek, 1940-41). Clerc and Sterne (1939) have used F1262 in a dose of up to 0.2 gm orally per day in a number of cases of angina pectoris and of disorders of rhythm with promising results.

There is only one outstanding exception to this agreement between results obtained on the rabbit auricle and in experimental fibrillation. Wégria and Nickerson found that adrenaline (in very large doses, 0.9 to 4.0 mg for dogs

averaging 10 kg in weight) increased the threshold to ventricular fibrillation induced by applying a short DC shock during the 'vulnerable period' of late systole. In my experiments adrenaline, even in a concentration of 1/25,000, increased the maximal rate of the auricle, this is in better agreement with the known physiological actions of adrenaline upon the heart, and its notorious effect (in quite small doses) of precipitating ventricular fibrillation in a heart which has been damaged by light chloroform, benzol or cyclopropane anaesthesia. In his reviews on the subject, Meek (1940-41) discussed the factors which may be involved in the latter phenomenon; he emphasized the evidence "that adrenaline strongly excites the automatic ventricular tissue of a heart already rendered highly irritable by chloroform". Orth, Leigh, Mellish and Stutzmann (1939) found that whereas sympathomimetic amines which contain a catechol nucleus (such as adrenaline, arterenol and cobefrin) produced multifocal ventricular tachycardia in dogs under light cyclopropane or chloroform anaesthesia, other amines such as ephedrine or neosynephrin were almost inactive in comparable doses. The presence of meta- or para-hydroxyl groups in the ring were not essential for the reaction, but they did very greatly increase its intensity. In the same way, while adrenaline would only increase the maximal rate of the auricle, the outstanding effect of ephedrine was to decrease it. The observation that adrenaline acts upon the heart muscle in a way directly contrary to the specific effect of quinidine, makes it easier to understand how adrenaline can precipitate ventricular fibrillation. Otto and Gold (1926) have described an interesting case in which adrenaline induced attacks of paroxysmal auricular tachycardia indistinguishable from those occurring spontaneously, under quinidine administration spontaneous attacks did not occur, nor could they be induced by adrenaline.

Acetylcholine also increased the maximal rate of the auricles. Acetylcholine, and particularly acetyl- $\beta$ -methylcholine, were observed to produce auricular or ventricular fibrillation on topical application or injection into experimental animals (Iglauer, Davis and Altschule, 1941, Smith and Wilson, 1944) and into human beings predisposed by thyrotoxicosis (Nahum and Hoff, 1940). Since large doses were used, adrenaline released from the adrenals or locally (Hoffmann, Hoffmann, Middleton and Talesnik, 1945) may have been an additional factor.

### *Structure and Action*

Inspection of Table I shows that most of the substances tested contain both an aromatic and a basic group. Nos 19 and 31 contain eleven and eight carbon aliphatic chains respectively in place of an aromatic ring, and they are both relatively inactive. No 29 with two phenyl rings is considerably more active than No 30, in which the rings are saturated. Sparteine is another example of a saturated ring compound which retains the characteristic activity. As to the nature of the aromatic rings, a comparison of Nos 14, 15, 16, 17 and 18 suggests that naphthalene is as good as diphenyl, and better than phenyl or

methoxyqumoline The benzilic ester series (22-26) is also particularly active, but trasentun, which also contains the diphenylmethane unit of structure, is relatively feeble

An increase in the number of carbon atoms attached to the basic nitrogen group is accompanied by an increase in activity in the benzilic ester series (22-25), there is also an optimum in the series 10-14, of which the di-*n*-propyl member was unfortunately not available Trefouël, Strickler and Bovet (1939) studied the ability of various homologues of F1262 to protect the ventricle of anaesthetized rabbits against fibrillation induced by an alternating current. The method was not strictly quantitative, but there was evidently an increase in activity as the number of carbon atoms attached to the nitrogen was increased up to the diethylamino-derivative (F1262), which was a little more active than the dimethylamino and dipropylamino compounds Chen, Wu and Henriksen (1929) also found, in a series of homologues of adrenaline and ephedrine, that an increase in the number of carbon atoms attached to the nitrogen or to the  $\alpha$ -carbon atom of the side chain caused an increase in the depressant action on the frog's heart and a change from pressor to depressor action in the pithed cat, their results also suggest a concomitant increase in toxicity on intravenous injection into rabbits Similarly Lands, Lewis and Nash (1945), studying the comparative pharmacological actions of some phenyl-, cyclohexyl- and cyclopentyl-alkylamines, found that increasing the size of the alkyl groups on the nitrogen from dimethyl to diethyl produced compounds that were depressor instead of pressor and had less accelerator action upon the heart.

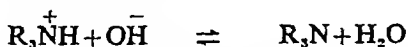
The nature of the linkage between the aromatic and basic groups does not appear to be of the first importance, it may be a carbinol, keto, ester or ether group or even a short alkyl chain as in methedrine, amphetamine and  $\beta$ -phenylethylamine Tréfouël, Strickler and Bovet (1939) found that a two-carbon chain was better than a three-carbon chain in compounds of the F1262 type Papaverine, pethidine and phenacaine provide yet more complex variants The more active compounds contain an aromatic (hydrophobic) and a basic (hydrophilic) group, and there is evidence that increased lipid-solubility is associated with increased quinidine-like activity (e.g., 14, 15, 16, 18), as it is with local anaesthetic activity (MacIntosh and Work, 1941) Similarly, Barger and Dale (1910-11) found in a series of aliphatic amines that in the higher members of the series (which are more lipid soluble) the pressor action on the spinal cat was complicated by a depressant action on the heart. The view that the basicity of the common local anaesthetics is of considerable importance in determining their activity was confirmed by the observation of Trevan and Boock (1927) that there was a linear relationship between pH and the logarithm of the minimal effective concentration applied to the rabbit's cornea This relationship supported the view that the active constituent of a solution of a local anaesthetic is the free base and not the ion or undissociated salt. This is probably true also for the quinidine-like action of drugs upon the heart not only are the most powerful

local anaesthetics most active upon the rabbit's auricle, but conversion into the quaternary salts (thus stabilizing the cation) of compounds 22 and 23 abolished their quinidine-like activity, just as conversion of local anaesthetics into quaternary salts abolishes their local anaesthetic activity

### *The Pharmacological Actions of Quinine, Quinidine and Procaine*

It is very remarkable that quinine, quinidine and procaine antagonize the effect of acetylcholine on many different types of tissue. They reduce its effect upon the rate and amplitude of contraction of heart muscle, and upon the isolated intestine. Harvey (1939, *a, b*) showed that the response of normal and denervated mammalian striated muscle to injected acetylcholine was reduced or abolished by quinine and procaine, procaine also abolished the response of the superior cervical ganglion to acetylcholine. Oester and Maaske (1939) obtained similar results to Harvey on striated muscle, and Frank, Nothmann and Hirsch-Kauffmann (1920) and MacGregor (1939, *a*) found that cocaine and procaine reduced the contractures caused by acetylcholine or nicotine in denervated mammalian muscle. Cocaine and procaine also reduced the pressor response to acetylcholine or nicotine in atropinized cats (MacGregor, 1939, *b*). Quinine inhibited the secretory action of choline or acetylcholine upon the salivary gland (Stavraky, 1932).

It may be observed that large quantities of these drugs are required to antagonize acetylcholine. The more specific antagonism towards acetylcholine in highly selective sites manifested by curare-like substances is a common property of *quaternary* ammonium salts, similarly among both the belladonna alkaloids and synthetic atropine substitutes the *quaternary* metho-salts are more active than the tertiary bases (Ing, Dawes and Wayda, 1945, Bülbring and Dawes, 1945). Whereas local anaesthetic and quinidine-like properties appear to be characteristic of the free base (disappearing or being greatly reduced when the tertiary base is converted into the quaternary metho-salt), curare-like and atropine-like properties appear to be characteristic of the cation (increasing when the tertiary base is converted into the quaternary metho-salt). Any solution of a tertiary alkamine such as quinidine or procaine will contain both the tertiary cation  $R_3NH^+$  and the base  $R_3N$  in equilibrium



so that it is not surprising to find that quinidine and procaine not only have local anaesthetic activity and a quinidine-like action upon the heart, but also a curare-like and atropine-like action in high concentrations. This conception of a solution of procaine as consisting of two dissimilar molecular species is of assistance in understanding its very complex action at neuro-muscular junctions. Its most striking action at this site is antagonism to acetylcholine, whether injected or released by stimulation of the motor nerve. This curariform action is, however, not sufficient to explain all the observed effects, Harvey (1939, *b*) showed that

procaine reduces the output of acetylcholine from the superior cervical ganglion on stimulation of the preganglionic nerve, and both Harvey (1939) and Jaco and Wood (1944) suggest that procaine also depresses the production of acetylcholine at the neuro-muscular junction by a 'local anaesthetic' action on the motor nerve endings. In addition, procaine has an action upon the muscle itself, in small doses occasionally causing increase, in larger doses decrease of directly excited twitches (Harvey, 1939, *b*, Macgregor, 1939, *a*). Of these effects the curariform is regarded as characteristic of the cation, and the direct depressant action upon nerve and muscle as characteristic of the free base. A further justification for this view is to be found in the analogous experiments of Harvey (1939, *a*) upon quinine. In this instance the direct action of the alkaloid upon the muscle was relatively greater, the prolongation of the refractory period, which was a prominent feature of this action, provides an obvious analogy with the action of these drugs upon cardiac muscle.

Although the typical properties of atropine appear to be a characteristic of the cation because they are more marked in atropine metho-salts, atropine itself is an ester of an aromatic acid and a tertiary alkamine, and might therefore be expected to show local anaesthetic and quinidine-like properties too. In high concentrations it was found to have a quinidine-like action upon the isolated rabbit auricle. It is also reputed to have a feeble local anaesthetic action. Brown (1937) has suggested that atropine may reduce the liberation of acetylcholine at the neuromuscular junction, since injection of 0.1 c.c. of 1:1,000 into the frog gastrocnemius abolished the response to nerve stimulation, but left a large part of the response to injected acetylcholine. Büllbring (1946), working with the isolated phrenic nerve diaphragm preparation of the rat, was driven to a similar conclusion, and has pointed out the qualitative resemblance between the actions of atropine and procaine on the neuromuscular junction. This can be accounted for by the fact that both are tertiary alkamine esters, and will therefore possess the properties characteristic not only of the action but also of the free base.

The difference between the pharmacological actions of the cation and of the free base is probably due to the inability of the former to penetrate inside cells. Thus curariform and atropine-like effects would be expected to occur at the cell surface (*cf* Cook, 1926), while local anaesthetic and quinidine-like properties would be dependent on the ability of the free base to penetrate the cell membrane. (Presumably quaternary compounds possessing atropine and curare-like properties act at the junction between nerve and effector tissue, because only there can they come into contact with the 'transmission process', whatever that may be). While this difference between cation and free base implies a considerable limitation of the site of action of the two molecular species, and so of their pharmacological properties, one cannot help being impressed by the broad structural similarity of drugs which on the one hand antagonize the action of acetylcholine at sites more or less strictly delimited, and

on the other hand depress the transmission of excitation in cardiac muscle and nerve (It goes without saying that this discussion only applies to the broad outlines of structure and action in the series of alkamines under consideration, it remains to be seen why curare, for instance, does not affect the action of acetylcholine on the heart, and why atropine has such an inconsiderable action at the neuro-muscular junction)

There is one further consideration which may be mentioned. The free base of a tertiary alkamine, having once penetrated the nerve or muscle cell, will come into equilibrium with its cation again according to the reaction given above. In this way it is theoretically possible for cations of tertiary bases to reach the inside of nerve and muscle cells. Hitherto the suggestion has been made that it is the free base of local anaesthetics and of quinidine substitutes which is the active constituent, it is conceivable, however, that the free base only acts by facilitating the entrance of the cation.

#### SUMMARY

1 A number of compounds have been tested as substitutes for quinidine upon a preparation of isolated rabbit auricles. Many of the local anaesthetics and spasmolytics in common use possess quinidine-like properties when tested in this way.

2 The most promising synthetic quinidine substitute is the benzilic ester of piperidino-ethanol (No. 25), which is 5.4 times as active as quinidine and has a therapeutic efficiency index from three to six times that of quinidine, according to whether their toxicities are compared in mice after intravenous or intraperitoneal injection respectively. This compound is considered worthy of therapeutic trial in man.

3 The relation between structure and quinidine-like action is discussed. The most active compounds possess aromatic and basic groups joined by ester, ether, keto or carbinol linkages. Within certain limits increase in lipid solubility and increase in the size of the alkyl group attached to the basic nitrogen atom are associated with increased activity.

The best local anaesthetics on the whole possess the greatest quinidine-like activity, and, as with local anaesthetics, the quaternary salts of very active tertiary compounds are quite inactive. This suggests that the active component of a solution is the free base rather than the cation.

4 While local anaesthetic and quinidine-like properties are characteristic of the free base (which can penetrate the cell-membrane), curariform and atropine-like properties appear to be characteristic of the cation (which, it is believed, acts at the cell surface). A solution of the aromatic ester of a tertiary alkamine such as procaine will contain both cation and free base in equilibrium. This conception of a solution of procaine as being composed of two dissimilar molecular

species is of some assistance in understanding its complex action upon the mammalian nerve-muscle preparation

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# THE ASSESSMENT OF ANALGESIC ACTIVITY IN NEW SYNTHETIC DRUGS

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Many methods for the measurement of analgesic effect have been described recently. They may be classified into mechanical pressure methods as described by Eddy (1928, 1932), electrical stimulation methods used by Koll and Reffert (1938), Macht and Macht (1940) among others, and lastly heat stimulus methods, which have been employed extensively in recent years by Hardy, Wolff and Goodell (1940).

The method of electrical pain threshold determination of Macht and Macht may have some quantitative value. Electrical stimuli from an induction coil are applied to the scrotal sacs of a rat, and when the stimulus is sufficiently strong to produce a sensation of pain the animal squeaks. If, however, the animal is heavily narcotized the stimulus may be high enough to contract the cremaster muscle and compress the testes without causing signs of pain. An attempt was made to use this method with an electronic stimulator, measuring the intensity of the stimulus by means of a peak volt-meter, but the contact resistance at the electrodes was too variable for the method to be reliable and it was not pursued further.

The method described by Hardy, Wolff and Goodell was next examined, but with human subjects our results did not accord with theirs. The apparatus employed was exactly as described by these workers with the single exception that absolute values of the radiant heat stimulus were not measured. A volt-meter and ammeter were connected to the lamp so that the power being dissipated as heat could be calculated. This presented no disadvantage as we were only interested in the change of pain threshold in the course of several hours. Day-to-day changes such as blackening of the lamp bulb and deposit on the lens system are therefore of no importance in this application. The ammeter was

omitted after a calibration curve had been prepared correlating the voltage across the lamp with the power dissipated in the lamp in watts

Wolff, Hardy and Goodell (1940), in a second paper, described some findings with morphine and codeine and their effects upon the pain threshold of man. They give time-action curves for morphine sulphate with doses of 0.1 mg to 30 mg for man. We have been unable to reproduce these curves, and even when 10 mg of morphine was injected into human subjects, with the actual drug unnamed, the rise in pain threshold was not statistically significant. A dose of pethidine of 100 mg by mouth in several subjects caused a slight elevation of the pain threshold, but the recognition of the actual threshold intensity became a very difficult matter. Similar difficulties have been experienced by Dodds, Lawson, Simpson and Williams (1945) in testing diphenylethylamine compounds for analgesic action in the human subject. For ourselves we cannot say that therapeutic doses of morphine or pethidine raise the pain threshold as determined by the apparatus of Hardy et al., but we did find it increasingly difficult to discern a critical end-point after the doses of the drugs mentioned above. It may be that we were ignoring the point of obvious pain sensation and concentrating too much on the detection of a minimal sensation, but it was usually a most uncertain method.

The method of Hardy, Wolff and Goodell, however, gives clear-cut values for changes in the pain threshold which result from ischaemia, as shown in Fig 1. The pain threshold of a male subject, who had previously shown a high degree of discrimination for small changes in the intensity of the radiant heat stimulus, was determined three times at ten-minute intervals and found to be constant. A sphygmomanometer cuff was then put on the right arm and inflated to a pressure of 200 mm Hg, and the pain threshold was determined at intervals of

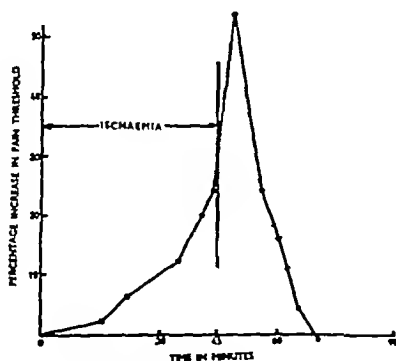


FIG 1—The effect of local ischaemia on the pain threshold in man

approximately ten minutes. After forty-five minutes the pressure in the cuff was released and the pain threshold determinations continued until the values obtained were similar to those at the commencement of the experiment.

It will be seen that there is a real rise in the level of threshold stimulus, and this rise became much more rapid immediately after the sphygmomanometer cuff had been deflated, at the same time the subject complained that the pain was much more intense in the arm as a result of the resumption of blood flow than it was at any time during arterial occlusion. In this case the end-point of the pain threshold determination was quite well defined. In the experiments described by Hardy, the pain threshold started to fall as soon as the circulation was restored.

In view of the difficulty of detecting a precise end-point when drugs have been administered to man, and more especially since the toxicities of many of the substances we had to examine were not well established, it was decided to try the apparatus on various small laboratory animals.

The first animal used was the guinea-pig, and a shaved and blackened area of the flank was used for the radiant heat stimulus. Since the aperture employed by Hardy was too large in this case, and in view of their evidence that the greater the area of skin stimulated the greater is the heating sensation, whereas there is no similar area summation of pain sensitivity, the area of the aperture was altered to 1 sq cm. Since guinea-pigs are excitable and did not give a precise end-point their use was abandoned and attention was turned to the use of rats.

Rats were first used in the same way as guinea-pigs, the pain threshold was indicated by a flinching reaction of the rat in which there is a general quivering of the body just before the shutter stops the radiant heat stimulus. At this point our attention was drawn to the work of D'Amour and Smith (1941), in which rats were used in a similar apparatus, but the tip of the tail was used as the sensory area. The method differs somewhat from that of Hardy, as the time of exposure to a set intensity of radiation is the adjustable factor and is timed with a stop-watch. The differences observed are small and need some automatic device to eliminate the reaction time of the observer. We endeavoured to do this by allowing the rat tail to lie in a groove in a board and across the tail was placed a lightly spring-loaded contact strip dipping in a mercury cup arranged to stop an electric chronometer if the tail was withdrawn.

The method did not give such consistent results as the use of rats with the Hardy apparatus mentioned above and was therefore abandoned. In the course of these investigations, however, we found that the rat tail was a very sensitive area and more suitable for stimulation than the flank. The Hardy apparatus was therefore modified by fitting a platform at right angles to the radiant heat aperture so that the rat could be placed upon it with its tail in front of the 1 sq cm aperture. The duration of stimulus was decreased to 2 secs so that the heating effect would be less and the shutter was adjusted to operate silently once in 15 seconds. This apparatus is drawn diagrammatically in Fig 2.

The rat, its tail blackened with Indian ink, is placed upon the platform with the tip of the tail across the aperture and the operator restrains it gently with

his right hand. The stimulus intensity is adjusted by the variable resistance controlling the lamp current and a sufficient number of trials are made, usually 4 to 6, to enable the operator to ascertain the pain threshold. The detection of the pain threshold stimulus is not a simple observation, and it is necessary to

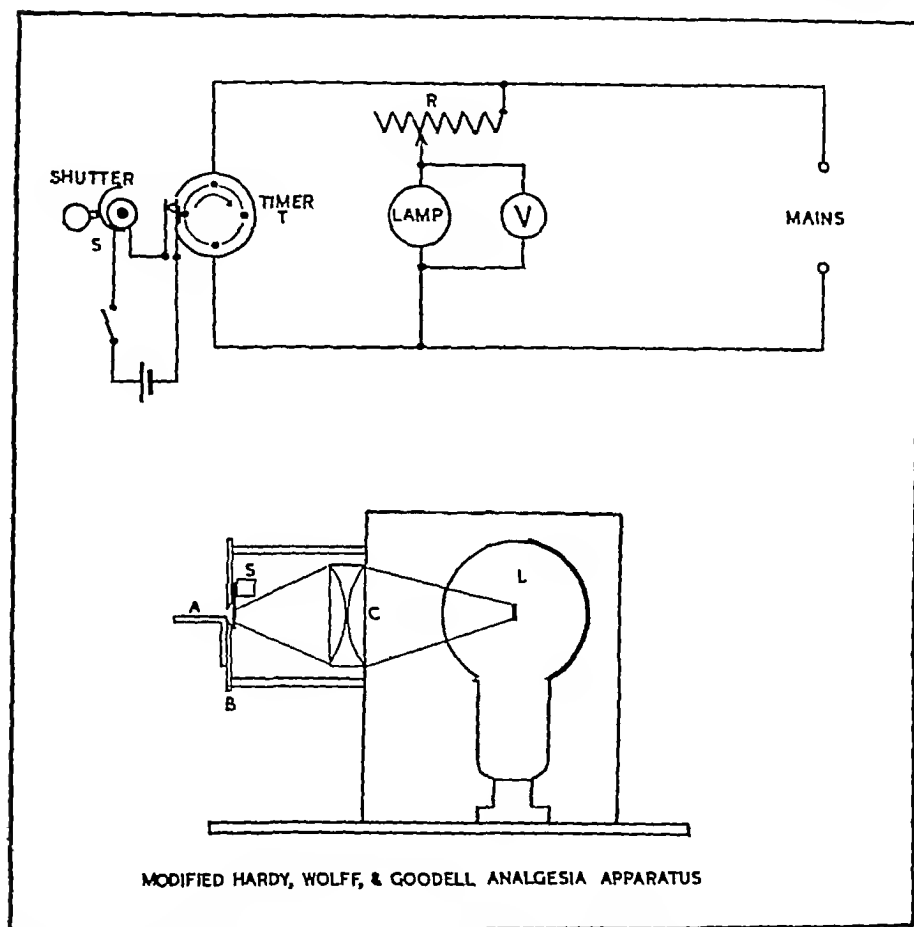


FIG 2.—The apparatus of Hardy, Wolff and Goodell (1940) modified for use with rats

*Upper diagram* Electrical circuit. R, resistance to reduce the voltage across the lamp to  $1/3$  of its rated value when fully in circuit (50 ohms in this apparatus using a 200 volt lamp). T, timer for magnetic shutter, this consists of a synchronous clock motor with a four point cam so that the shutter is lifted for 2 secs at intervals of 15 secs. S, shutter, made from an old domestic electric bell indicator and operated on a 1.5v dry cell.

*Lower diagram* A, small shelf to support the rat with the tip of the tail across the radiant heat aperture. B, paxolin front panel now fitted with water cooling as described in the text and carrying the shutter, S. C is a lantern condenser of 4.5 in diameter and about 10 cm focal length. L, projection lamp with grid filament, 1,000 watts, mirror-backed.

allow the operator to become sufficiently experienced before tests are conducted. Furthermore, it has been found that it is necessary to train new rats to discriminate between stimuli of different intensity before using them for the test. This is done by allowing several successive stimuli of high intensity to fall upon the tail until the rat removes its tail in a positive manner after each stimuli. The stimuli are repeated at a slightly lower intensity and the rats are then able to discriminate sufficiently accurately. This training is only necessary with rats which have not been used before for these experiments on the same day. If the necessity of training the rats is not appreciated the determination of the initial pain threshold becomes very time-consuming. No single response is used as a criterion, but a general assessment is made from slight movements of the tail, quivering of the body of the rat, or in cases of excessive stimulus removal of the tail. If the stimulus is below the threshold, no change in the rat's activity will be observed.

A recent paper by Slaughter and Wright (1944) draws attention to the need for stabilization of the electricity supply to the apparatus and the use of a thermocouple to measure the temperature of the surface being stimulated. Stabilization is not necessary in our case, because the stimulus is measured as the product of the instantaneous readings of the lamp current and voltage. We were not able to show that the temperature of the radiant heat upon a thermocouple had any advantage over our purely electrical measurements when each experiment was self-contained and completed in one day.

The method described above has been adopted in these laboratories and has proved very satisfactory for assessing the presence or absence of analgesic activity in new drugs and giving a quantitative comparison between them.

The method of pain threshold determination having been established it was necessary to investigate a suitable experimental design to enable the maximum information to be obtained. Rats are convenient in this respect, as all our animals are a pure Wistar strain and it is easy to use litter-mates in such an experiment. A series of preliminary experiments was made to find out whether there is a greater variation in the response of rats to analgesic drugs when they are not taken from the same litter than when litter-mates are used. It was also hoped to discover whether the "cross-over" construction of a test would result in a greater consistency of response.

The experiments were arranged as follows

### *Experiment I*

Three sets of three rats, not litter-mates, were used and the experiment was arranged as a three-way cross-over test on three days so that each set of three rats received different doses of morphine on each day and had each received all three doses at the conclusion of the experiments.

TABLE I

EXPERIMENT I THREE-WAY CROSS-OVER TEST ON RATS WHICH WERE NOT FROM THE SAME LITTERS  
DAY 1

Rat No	1	2	3	4	5	6	7	8	9
Initial pain threshold W	268	259	230	222	248	222	213	213	222
Morphine dose	2.5 mg./kg.			3.0 mg./kg.			3.5 mg./kg.		
Pain threshold after 30 min. W	279	277	307	286	268	320	279	286	320
Response %	+20	+8	+33	+30	+8	+44	+31	+34	+44

DAY 2.

Initial pain threshold W	230	279	237	222	230	237	259	213	279
Morphine dose	3.5 mg./kg.			2.5 mg./kg.			3.0 mg./kg.		
Pain threshold after 30 min. W	351	286	259	342	259	230	259	230	268
Response %	+53	+3	+3	+54	+12	-4	0	+8	-4

DAY 3

Initial pain threshold W	237	268	230	222	237	213	237	222	230
Morphine dose	3.0 mg./kg.			3.5 mg./kg.			2.5 mg./kg.		
Pain threshold after 30 min W	307	307	286	351	320	334	230	279	259
Response %	+25	+15	+25	+58	+35	+57	-4	+25	+13

Response % is a term used for the percentage change in the initial pain threshold after treatment with analgesic agents

MEAN RESPONSES OF ALL RATS.

Dose mg./kg.	Response %
2.5	+17.6
3.0	+16.7
3.5	+35.3

The actual results obtained are reproduced *in extenso* in Table I to show the general type of record obtained. We had previously found that the peak of analgesic action following the injection of morphine into rats occurred at a quarter to half an hour after injection, and consequently the reading was taken for each rat half an hour after injection

Table II shows an analysis of these results, and it will be seen that there was a significant difference in response between the doses of morphine of 0.3 and 0.35 mg/100 g but not between 0.25 and 0.3 mg/100 g. This is probably due to all three dose levels not lying on the steepest part of the dose response curve.

TABLE II  
ANALYSIS OF RESULTS RECORDED IN TABLE I

	2.5	Dose, mg./kg. 3.0	3.5
Mean Response	17.6	16.7	35.3
Variance of mean response	37.5	23.0	48.4
Standard deviation	6.1	4.8	6.9
Response difference		0.9%	18.6%
Variance of mean difference		60.5	71.4
Standard deviation		7.8	8.4
"t"		1.17	2.21
Degrees of freedom		16	16
"p"		0.9	0.04

### Experiment II

A similar experiment, using litter-mates in such a manner that each set of rats had the same litter history as the other two sets, was next performed. It will be seen from the results in Table III that a difference in dose of 0.5 mg/100 g

TABLE III  
ANALYSIS OF RESULTS OBTAINED IN EXPERIMENT II IN WHICH LITTER-MATE RATS WERE USED

	2.5	Dose, mg./kg. 3.0	3.5
Mean response	3.8	23.2	33.6
Variance of mean response	20.8	8.7	75.0
Standard deviation	4.6	2.9	8.7
Response difference		19.4%	10.4%
Variance of mean difference		29.5	83.7
Standard deviation		5.4	9.1
"t"		3.6	1.14
Degrees of freedom		16	16
"p"		0.005	0.25

was distinguished at the lower end of the dose scale but not at the higher level. It can be concluded that these rats were probably more sensitive than those of Experiment I.

The mean variance is, however, very much the same as that of the previous experiment, so that it does not seem that litter-mate rats are superior to mixed rats from the same stock.



*Experiment III*

In this experiment three sets of nine rats with litter-mates in each of the three sets were used on the same day. The results given in Table IV show that there was discrimination between each pair of doses, but the dose difference of 0.05 mg/100 g was too close for the difference in response to be statistically significant with such a small number of animals. The mean variance was rather less than in the other two experiments.

TABLE IV  
ANALYSIS OF RESULTS OBTAINED IN EXPERIMENT III IN WHICH LITTER-MATE RATS WERE USED ON THE SAME DAY

	Dose, mg./kg.		
	2.5	3.0	3.5
Mean response	14.0	18.0	25.0
Variance of mean response	4.4	13.4	32.0
Standard deviation	2.1	3.7	5.6
Response difference	4%	7%	
Variance of mean difference	17.8	45.4	
Standard deviation	4.2	6.73	
"t"	0.953	1.04	
Degrees of freedom	15	16	
"p"	0.3	0.3	

In the absence of any outstanding differences in the results, it was concluded from these experiments that the use of litter-mates did not give any great increase in accuracy to the test, and the advantage of using a cross-over arrangement was not particularly apparent. Although there is apparently no outstandingly desirable form for the experiment, we find it more convenient to use some form of cross-over test where any high degree of accuracy is required, as the work is thus divided over several days. If only approximate indications of analgesic potency are required a comparison is usually made between two litter-mate groups on the same day.

#### THE DURATION OF ACTION OF MORPHINE ON RATS

Although some previous experiments had indicated that the peak of analgesic action of morphine in rats occurred about half an hour after injection an experiment was performed in which the pain threshold was determined at frequent intervals after injection of morphine.

Two sets of six rats were used, the rats of one set being litter-mates of the corresponding rats in the other set. All rats were given 0.35 mg/100 g of morphine by subcutaneous injection after the initial pain threshold readings had been taken. One set of rats was used for pain threshold measurement after a quarter, half, three-quarters and one hour from the time of injection and the second set for readings at 10 minutes, 20 minutes, and 35 minutes.

It was not possible to use the same rats at all these time intervals as the actual readings take about 10 minutes for six rats. The following results were obtained

*Set I*

Time after injection, minutes	15	30	45	60
Actual percentage rise of threshold	23.4	13.8	12.4	-3.5

*Set II*

Time after injection, minutes	10	20	35
Actual percentage rise of threshold	4.6	17.9	2.8

This experiment shows that the peak of morphine analgesic action occurs after about 15–20 minutes in rats and this time interval is now employed for drug comparisons with morphine together with a time-response determination for the drug under examination.

We had noticed on several occasions that the front panel of the apparatus in which the light aperture was cut became increasingly hot when readings were taken at such frequent intervals that the lamp was left on for long periods. This produced an apparent lessening of the analgesic effect after the initial reading because less radiant heat was needed to elicit a response. This was observed especially when control rats were incorporated in a test. The mean pain threshold of a group of such rats usually appeared to fall by some 10 per cent after the initial reading.

Since the above experiments were done a new front panel has been made for the apparatus. This was constructed from paxolin sheet 1/4 in thick. An annular recess was milled out in the side facing the lamp and surrounding the stimulus aperture. The recess was 1 in wide and 3/16 in deep, and the rear of the panel was faced with a piece of 1/16 in paxolin, cemented and screwed over the annular recess and having an aperture in its centre coinciding with the stimulus aperture. Two thin tubes were fitted in the edge of the panel so that a stream of water could be passed through the annular space. With this device the temperature of the panel remained very constant even after the lamp had been on for a period of some hours.

In addition to this we have found that experience of the method enables the end-points of the threshold determination to be found more accurately, and consequently smaller differences in response assume more significant proportions.

## DOSE RESPONSE CURVE FOR ANALGESIC ACTION OF MORPHINE IN RATS

Six litters of rats were used with five or more rats in each group, and so grouped that one rat from each litter was placed into each of the five groups. The rats were then given doses of morphine of 0.27, 0.30, 0.33, 0.36, and 0.40 mg/100 g to each group respectively and the pain threshold change determined as a percentage of the initial value after 20 minutes.

TABLE V

RESULTS OBTAINED IN AN EXPERIMENT TO ESTABLISH A DOSE/RESPONSE RELATIONSHIP FOR THE ANALGESIC ACTION OF MORPHINE

Group	Dose of Morphine, mg./kg.	Percentage increase in pain threshold for individual rats						Mean Response
		1	2	3	4	5	6	
1	2.7	25	32	20	16	21	17	21.8
2	3.0	16	18	8	38	39	20	23.1
3	3.3	54	36	66	43	23	38	43.0
4	3.6	57	89	74	82	66	66	72.3
5	4.0	97	91	85	77	83	72	84.1

The results obtained are given in Table V. When plotted on a linear scale the mean response values gave the curve of Fig 3, and when plotted in a

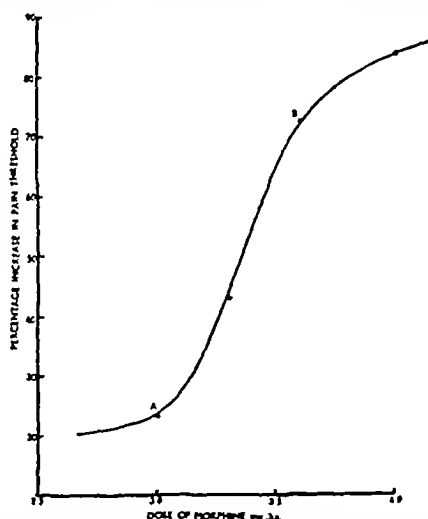


FIG 3—Curve relating the dose of morphine injected and the mean elevation of the pain threshold 20 minutes after injection. Each point is a mean value for six rats.

semi-logarithmic manner the usual formula to a regression-line of this type was applied to section AB and upon calculation the slope of this response was found to be 576.

Formula to log-dose/response curve

$$y = 48.9 + 576 (x - 0.483)$$

(by substitution in the formula  $y = \bar{y} + b(x - \bar{x})$ )  
therefore  $b = 576$

Several days later two of the groups of rats were taken at random and injected with two solutions of morphine, both made up to a strength such that

a dose of 10 cc /100 g would cause a response within the measurable limits of the method. The percentage increase in pain threshold was determined for each rat and the mean value calculated for each group

Group	Dose	Mean percentage increase in threshold
1	Soln.A 10 cc /100 g	20.6
2	Soln.B 10 cc /100 g	63.1

When these values were substituted in the equation and using the slope value of 576 the following results were obtained

	Soln. A	Soln. B
Log of estimated dose	= 1.465	1.543
Therefore estimated dose	= 0.292 mg	0.349 mg
Estimated Concentration of solutions	= 0.292 mg /cc	0.349 mg /cc
Limits of error $P=0.95$	= 0.265-0.312 mg	0.323-0.375 mg
Actual strengths of solutions prepared	= 0.30 mg /cc	0.36 mg /cc

It is very rarely that we have any need to perform an accurate assay of analgesic drugs, but it would seem that it is quite a practical procedure. Normally with new compounds it is sufficient to aim at obtaining approximately a 50 per cent increase in pain threshold in a comparison with morphine and then the relative activity of the two drugs can be roughly represented by the ratio of the doses employed.

A comparison was made between morphine and heroin to illustrate this method of approximate comparisons and results are given in Table VI. Since heroin is known to be a more effective analgesic than morphine a dose of 0.1 mg /100 g was given to nine rats and a dose of 0.35 mg /100 g of morphine to a set of nine rats of similar litter constitution.

It was found that the effect of the dose of heroin was such that the pain threshold level of most of the rats rose above the upper limit of the test stimulus range. The results are shown graphically in Fig. 4.

The experiment thus showed that heroin was more than 3.5 times as effective as morphine as an analgesic and a second experiment arranged as a cross-over test with two groups of nine rats was performed with doses of 0.05 mg /100 g and 0.35 mg /100 g of heroin and morphine respectively.

The mean results for eighteen rats are given in Table VI and graphically in Fig. 4. The graph indicates that the maximum analgesic action of morphine occurs one hour after injection. This is most probably not so and previous experiments have indicated that it does, in fact, occur between fifteen and forty-five minutes after injection. The readings at one quarter and one hour for the animals receiving morphine probably lie on either side of the time for maximal analgesic

action, so that the true point of maximal action is not seen in these curves. It is, of course, unsound to compare the analgesic response of a group of rats given a certain dose of a drug with another response from a different group of rats at a different time given the same treatment, especially in view of the very steep slope

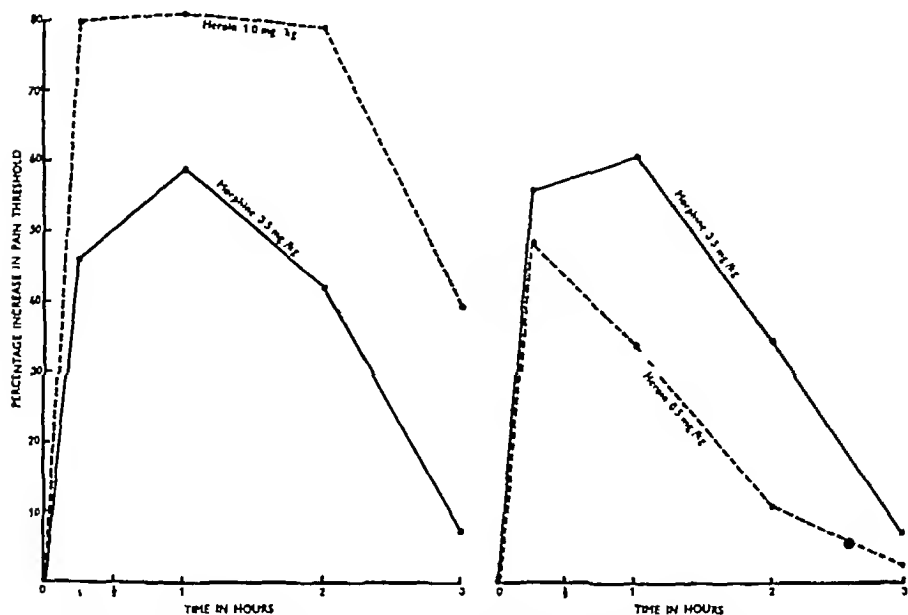


FIG 4—A comparison between the analgesic effects of morphine and heroin

*Left-hand curves* mean values obtained from groups of nine rats in each case

*Right-hand curves* mean values obtained from groups of eighteen observations at each point. Eighteen rats were used in the form of a cross-over test

of the log dose/response relationship usually given by analgesic drugs. It does seem, however, that these later experiments, performed after water-cooling had been fitted to the apparatus, show a bigger analgesic response for a given dose of morphine than do the earlier ones, a result rather to be expected.

It will be seen that heroin is rather less than seven times as potent as morphine as an analgesic, and that the duration of action is in both cases similar, which is supported by clinical experience.

From this experiment we may draw some general conclusions. If a test is arranged as a cross-over test using nine animals in each group and deriving eighteen observations on each treatment it is usually found that differences in the mean response percentages to each treatment exceeding ten are significant to  $P > 0.95$  limits.

The variance of the response is naturally higher at times when the analgesic action is near the centre of the dose/response curve, but it will be seen in the

TABLE VI

THE RESULTS OBTAINED IN AN EXPERIMENT TO COMPARE THE ANALGESIC ACTIONS OF MORPHINE AND HEROIN

Dose	Percentage increase in pain threshold (Mean value for 18 rats)			
	½ hr	1 hr	2 hr	3 hr
Morphine 3.5 mg./kg. S.C.	56	61	35	9
Heroin 0.5 mg./kg. S.C.	48.5	34	11	2
Difference in response %	7.5	17	24	7
Variance of mean difference in response	66.2	48.0	38.0	19.4
Standard deviation	8.14	6.9	6.16	4.4
"t"	0.922	2.46	3.9	1.592
Degrees of freedom	34	34	34	34
"p"	0.4	0.02	< 0.001	0.1

experiment that a difference of seventeen between the two treatments at the first hour was highly significant.

It has been shown that the method is suitable for the comparison of the analgesic action of new drugs and is at the same time suitable as the basis of a relatively exact biological assay. A dose of morphine should always be included as a standard of comparison for the examination of new drugs, and we also include a group of animals receiving both morphine and the new compound as separate injections at the same time, in order that potentiation phenomena may not be overlooked.

#### SUMMARY

1 A brief review of the methods for the measurement of analgesic action is given together with an account of experiments with the apparatus described by Hardy, Wolff and Goodell on human subjects. The results which these workers obtained with morphine could not be repeated, but their observation that local ischaemia causes an elevation of the general pain threshold was confirmed.

2 In order to test drugs of unknown toxicities for analgesic action the method has been modified for use with rats. The blackened tip of the tail is used as the sensitive area and the pain threshold is represented by the power of the lamp in watts.

3 After an initial training period the rat is able to distinguish small changes in the intensity of the applied stimuli, and using several sets of rats at the same time comparisons between analgesic drugs can be made. The slope of the log dose/response curve for analgesic drugs is very high and can be determined within the experiments when an accurate assay is required. Usually, however, it is sufficient to adjust the dose of the drugs so that approximately a 50 per

cent increase in the pain threshold results. The doses then represent the ratio of the analgesic actions of those drugs in inverse proportion.

4 Little advantage could be shown in the use of litter-mates or in the adoption of a cross-over arrangement. The experiments on these points did not show any outstandingly preferable arrangement and were therefore not continued to a point of statistical finality. It was found that dividing a group of animals equally among the drugs to be tested and repeating the experiment on a sufficient number of days for all the animals to have received all the treatments was a practical arrangement, since it is not possible to test a large number of rats at short time intervals.

5 As an example of the method a comparison was made between the analgesic effects of morphine and heroin, and heroin was found to be rather less than seven times as potent as morphine.

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## METHYL-THIOURACIL AND THIOURACIL AS ANTITHYROID DRUGS

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4-Methyl-2-thiouracil is beginning to replace 2-thiouracil in the treatment of thyrotoxicosis. It is cheaper and easier to prepare than thiouracil. Leys (1945) has reported the treatment of sixteen patients without any serious adverse effects and O'Donovan (1944) states that it is less toxic than thiouracil. Favourable preliminary results have also been obtained on a large number of patients in Denmark by Freiesleben, Kjerulf-Jensen, Meulengracht and Schmith (1944) and Jersild and Nissen (1944).

The present experiments were undertaken to compare the antithyroid activities of methyl-thiouracil and thiouracil fed to rats.

### METHODS

Seventy-five young male albino rats, 3 to 4 weeks old, were placed on Coward's diet and allowed food and drinking water *ad libitum*. Ten controls received Coward's diet only, 36 received the diet supplemented with 0.11 per cent methyl-thiouracil, and 29 the diet supplemented with 0.1 per cent thiouracil. (The molecular weights of methyl-thiouracil and thiouracil are in the proportion of 11 to 10). The rats were weighed twice weekly and at fortnightly intervals 1 control rat, 3 methyl-thiouracil rats, and 3 thiouracil rats were killed and the thyroids removed, weighed, and kept for histological examination. After 100 days on diet, most of the remaining rats were killed and the thyroids, adrenals, pituitaries, liver, kidneys, spleen, testes, and pancreas removed for histological study.

Metabolism experiments were carried out on 4 rats treated with methyl-thiouracil and on 4 treated with thiouracil after 23 weeks on diet. The water intake, food intake, and urine output were measured for each group of 4 rats over four consecutive 24-hour periods. The methyl-thiouracil and thiouracil contents of the urines were determined colorimetrically, using Grote's reagent. This reagent was prepared according to Williams, Jandorf, and Kay (1944). It was diluted 1 in 20 with 0.05 M phosphate buffer at pH 6.0 before use as advocated by Chesley (1944). Better colour development was obtained at this pH than at pH 8.5 to 9.0 used by Williams *et al* (1944). For the urine estimations, an equal volume of the dilute Grote reagent was added to a 1 in 20 dilution of the urine. The green colour developed was read in a photo-electric colorimeter, using a Wratten filter No. 29. The time required to attain full colour development varies considerably not only in different urine samples but also in standard methyl thiouracil and thiouracil solutions set up on different days. For this reason, it was found necessary to construct a calibration curve for methyl thiouracil and thiouracil each day and to take readings of both standards and urine samples at frequent intervals until constant values were obtained.



Metabolic rate determinations were carried out in a constant temperature room at 25° C. after a fasting period of 24 to 30 hours using, in principle, the closed circuit method of Regnault and Reiset (1943). Both  $O_2$  consumption and  $CO_2$  output were measured over a period of 45 minutes.

Respiration rate determinations were carried out on thyroids and diaphragm. The rats were killed by a blow on the head, the thyroids removed as quickly as possible, weighed,

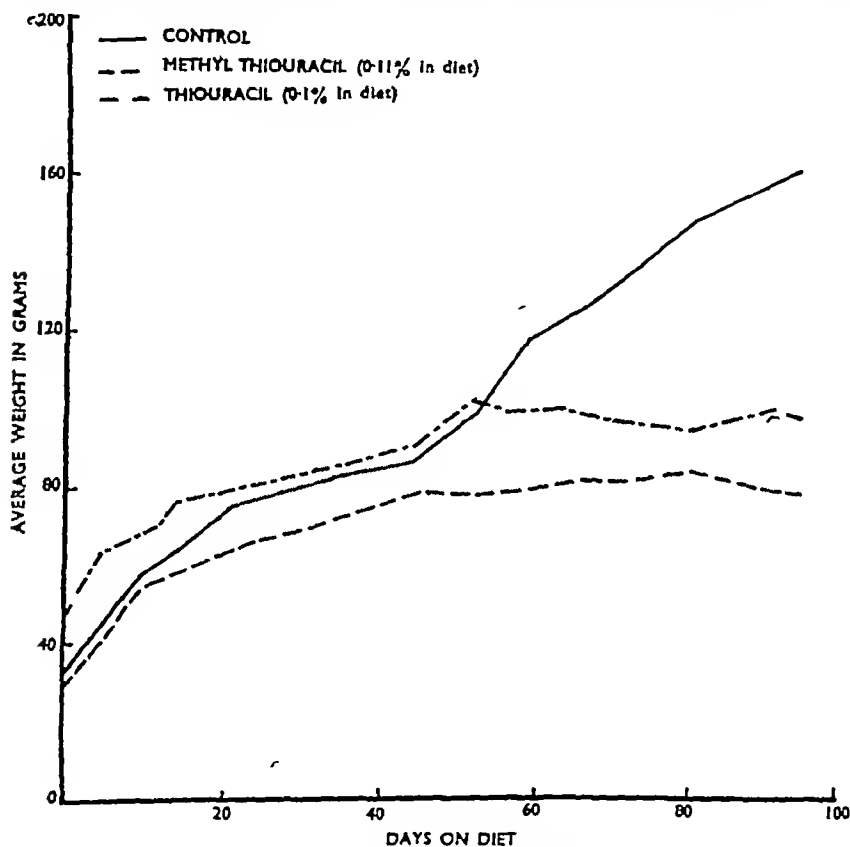


FIG 1 Growth curves of rats

cut into thin slices with scissors, suspended in 2 cc. Ringer phosphate at pH 7.4, and the  $O_2$  uptake measured in  $O_2$  at 37.5° C. in Warburg manometers over a period of 60 minutes. Since it was found necessary to use at least 50 mg wet weight tissue for each estimation, this necessitated using the thyroids from five or six control rats, one or two thiouracil rats, and three methyl-thiouracil rats. The diaphragms were cut in halves and one estimation carried out on each half.

The results were expressed on a wet weight basis.

## RESULTS

The growth curves of the three groups of rats are shown in Fig. 1.

Both methyl-thiouracil and thiouracil produce approximately the same inhibition of growth

The thyroid weights are given in Fig 2

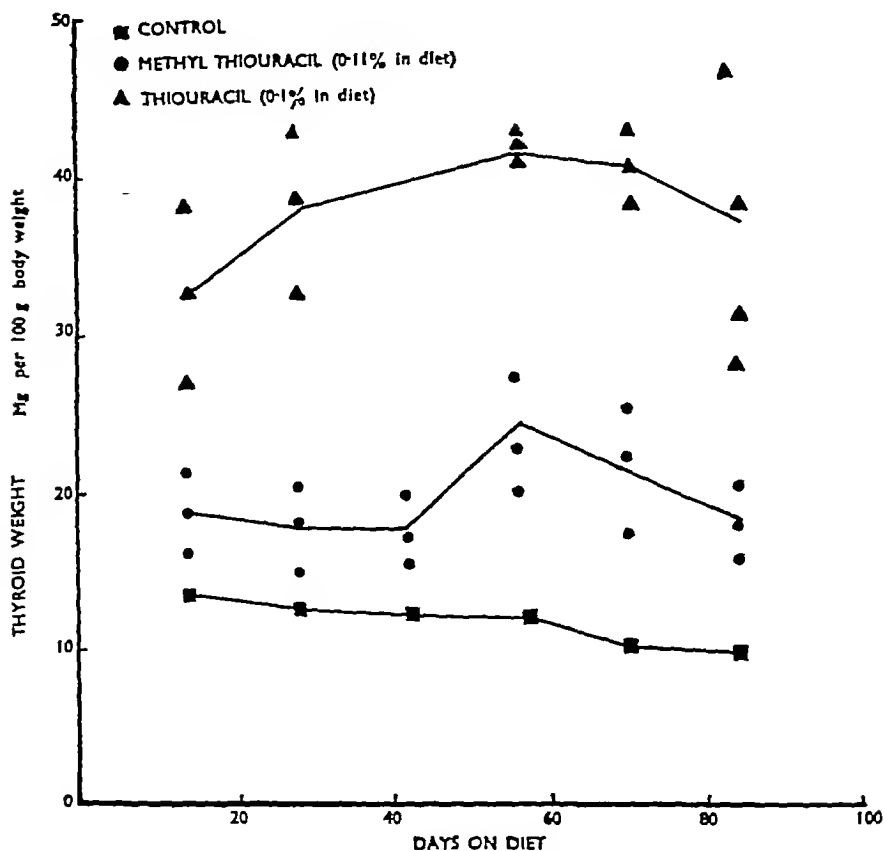


FIG 2. Thyroid weights of rats

All the thyroids of the rats treated with methyl-thiouracil were larger than those of the controls but smaller than any of the rats treated with thiouracil. The thyroids of the rats treated with methyl-thiouracil were of the same pinkish-buff colour as the controls, whereas those of the rats treated with thiouracil were extremely red and vascular. This greater goitrogenic effect of thiouracil is reflected in the microscopic appearance of the thyroids. The thyroids of the control rats (Fig 3) have regular-shaped, colloid filled alveoli lined by flattened epithelium. In contrast to this, the thyroids of the rats treated with thiouracil (Fig 5) have enlarged irregular-shaped alveoli containing no colloid and are lined by high cubical epithelium. Many of the epithelial cells are vacuolated. The

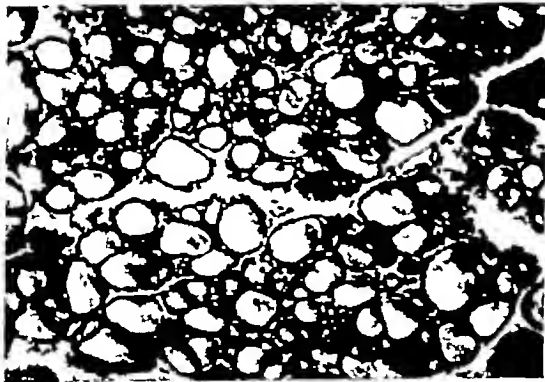


FIG 3 —Thyroid of control rat showing regular-shaped alveoli filled with colloid and lined with flattened epithelium

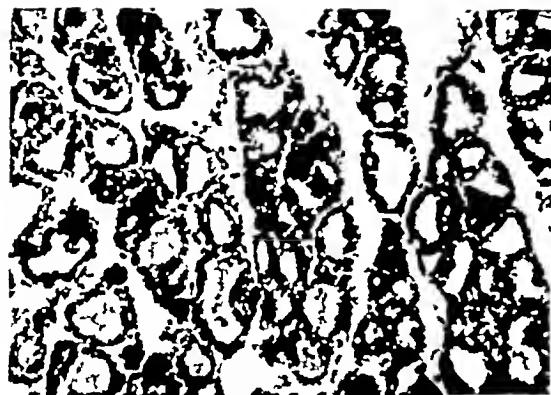


FIG 4—Thyroid of methyl-thiouracil treated rat showing alveoli lined with high cubical epithelium. Many alveoli contain vacuolated colloid



FIG 5—Thyroid of thioracil treated rat showing irregular-shaped alveoli lined with high cubical vacuolated epithelium. Very little colloid remains

Sections of rat thyroids stained with haematoxylin and eosin after 100 days on diet. (Magnification 105)

thyroids of the rats treated with methyl-thiouracil (Fig 4) are intermediate in structure. Although the alveoli are not so regular as the controls and the epithelium is high cubical, most alveoli still contain some colloid.

The greater cellular activity of the thyroids of the rats treated with thiouracil is also shown in the respiration rate of thyroid slices. Table I gives the results after the rats had been on diet for 24 weeks. The values for the respiration rate

TABLE I  
RESPIRATION RATE OF THYROID SLICES  
( $Q_{O_2}$ =cu. mm  $O_2$  at N.T.P. per mg. wet weight tissue per hour)

Control (C)	Methyl Thiouracil (MT)	Thiouracil (T)
-0.87		-1.57
-0.69		-1.50
-0.83		-1.37
-0.79	-1.02	-1.18
-0.80	-1.09	-1.50
		-1.29
Mean -0.79	Mean -1.055	Mean -1.40

Mean difference MT-C  $t=4.8$ ,  $n=5$ ,  $p=0.0048$   
T-MT  $t=3.0$ ,  $n=6$ ,  $p=0.025$

of the thyroids of the rats treated with methyl-thiouracil are intermediate between those of the controls and of the thiouracil-treated rats. The  $Q_{O_2}$  appears to be proportional to the degree of thyroid hyperplasia.

Neither thiouracil nor methyl-thiouracil has any effect on the respiration rate of diaphragm removed from rats fed these drugs. Values for the  $Q_{O_2}$  of diaphragm are given in Table II. This is very difficult to reconcile with the fact

TABLE II  
RESPIRATION RATE OF DIAPHRAGM  
( $Q_{O_2}$ =cu. mm  $O_2$  at N.T.P. per mg. wet weight tissue per hour)

Control	Methyl Thiouracil	Thiouracil
-1.35	-1.24	-1.34
-1.51	-1.34	-1.38
-1.41	-1.48	-1.52
-1.27	-1.27	-1.28
-1.42	-1.37	-1.38
-1.30	-1.27	-1.35
-1.29		-1.29
Mean and Standard Deviation } $1.36 \pm 0.0045$	$1.33 \pm 0.0065$	$1.36 \pm 0.0041$

that both drugs produce a considerable decrease in the metabolic rate and that in thyroidectomized rats the  $Q_{O_2}$  of skeletal muscle is reported to be greatly reduced (Dye and Maugham, 1929). Jandorf and Williams (1944) have also found that

feeding thiouracil to rats produces no alteration in the values for the  $Q_{O_2}$  of liver and diaphragm

Although thiouracil is only sparingly soluble in water (approximately 0.2 per cent at 20° C.), it is nearly five times as soluble as methyl-thiouracil. It was thought that the smaller goitrogenic activity of methyl-thiouracil might be due to incomplete absorption of the drug, and consequently some metabolism experiments were carried out. The results (Table III) clearly indicate that the major part of the methyl-thiouracil is absorbed, since approximately 62 per cent is excreted in the urine.

TABLE III  
METABOLISM EXPERIMENT ON FOUR METHYL-THIOURACIL AND FOUR THIOURACIL-TREATED RATS  
AFTER 23 WEEKS ON DIET

24-hr Period	Water Intake (cc.)	Urine Output (cc.)	Drug ingested (mg)	Drug excreted (mg.)	% Drug excreted*
METHYL-THIOURACIL GROUP (MT)					
1	42	14.5	27.5	16.0	58
2	40	10.5	27.5	16.4	60
3	35	17.5	33.0	23.7	72
4	37	9.5	22.0	12.3	56
THIOURACIL GROUP (T)					
1	40	22.5	25.0	17.4	69
2	57	18.0	25.0	19.7	79
3	40	17.0	30.0	20.0	67
4	44	14.0	25.0	18.0	72

\*Mean difference T-MT  $t=2.298$ ,  $n=6$ ,  $p=0.058$

The metabolic rates of a few rats have also been determined. These results are given in Table IV. Both methyl-thiouracil and thiouracil produce approximately the same depression in metabolic rate.

TABLE IV  
HEAT PRODUCTION OF FASTING RATS AT 25° C  
Calories per sq m body surface per 24 hr

Control (C)	Methyl Thiouracil (MT)	Thiouracil (T)
	642	
	533	
	520	
	633	632
832	687	650
890	557	656
838	635	585
930	591	600
Mean 872	Mean 600	Mean 625

Mean difference C-MT  $t=8.23$ ,  $n=10$ ,  $p<0.001$   
T-MT  $t=0.835$ ,  $n=11$ ,  $p=0.4$

Both thiouracil and methyl-thiouracil produce changes in organs other than the thyroid. After rats had been on diet for 100 days, the anterior pituitaries of both groups showed changes identical with or closely similar to those seen after thyroidectomy, that is, a considerable reduction in the number of acidophil cells and an increase in the number, size, and degree of vacuolation of the basophil cells. No differential counts of basophils and acidophils were undertaken, but there was no obvious difference between the anterior pituitaries of the two groups. Slight changes were also observed in the adrenals, but nothing approaching the gross changes in the terminal stages with thiourea and thiouracil, previously reported (Glock, 1944). In both groups the reticularis was congested, there was swelling of the superficial cells of the fasciculata, and a marked reduction in the sudanophil staining material, particularly in the fasciculata. The most marked difference between the appearance of rats treated with methyl-thiouracil and thiouracil was that the rats treated with methyl-thiouracil were sexually very immature. With three exceptions, the testes of this group of rats had not descended after six months on diet. The descended testes were normal in structure, whereas sections of the undescended testes showed that spermatogenesis was inhibited and the tubules considerably reduced in size and containing relatively few cells, this is similar to the appearance of undescended testes in man. In the thiouracil-treated rats the testes had invariably descended.

#### DISCUSSION

The antithyroid activity of new compounds is generally tested by ascertaining the increase in thyroid weight of rats administered these drugs for a fixed period. Although antithyroid activity is associated with some degree of thyroid hyperplasia, the present results show that these two effects are not quantitatively related, and that it is essential to determine the metabolic rate in order to be able to assess antithyroid activity. Thus, although both methyl-thiouracil and thiouracil produce approximately the same depression in metabolic rate, the degree of thyroid hyperplasia and vascularity is very much less with methyl-thiouracil than with thiouracil. In this connection, it is interesting to note that Ciereszko (1945), testing purified thyrotropic hormone on chicks, found that thyroid size gave no indication of thyrotropic potency. Recently, McGinty and Bywater (1945) have taken the total iodine content of the thyroid as an index of antithyroid activity.

Since the completion of these experiments, the results of Danish workers have become available. Freiesleben, Kjerulf-Jensen and Schmith (1945) and Jensen and Kjerulf-Jensen (1945) report methyl-thiouracil to be the most active goitrogenic compound they have tested. No data, however, are given for thiouracil. Their results differ in some respects from those obtained in this country and America, notably in the action of 2-thiobarbituric acid. Christensen (1945) found it to be inactive, whereas Astwood (1943) found it to be almost as active as thiouracil.

## SUMMARY

1 The antithyroid activities of methyl-thiouracil and thiouracil have been compared on rats

2 Both methyl-thiouracil and thiouracil produce approximately the same inhibition of growth and the same depression of metabolic rate

3 Methyl-thiouracil produces considerably less thyroid enlargement and vascularity than thiouracil. The greater cellular hyperplasia of the thyroids of the thiouracil-treated rats is reflected in the higher values of the respiration rate of thyroid slices

4 Thyroid size should not be taken as a criterion of antithyroid activity. Metabolic rate determinations are essential

5 The use of methyl-thiouracil in the treatment of human thyrotoxicosis is advocated, since, although it produces the same depression of metabolic rate as thiouracil, the degree of thyroid hyperplasia and vascularity is considerably less

## ACKNOWLEDGEMENTS

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# AUTOMATIC APPARATUS FOR PHARMACOLOGICAL ASSAYS ON ISOLATED PREPARATIONS

BY

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In a pharmacological assay on an isolated preparation the experimenter has to repeat at regular time intervals various operations requiring his continued concentrated attention. These include control of the drum, adjustment of the fluid level of the isolated organ bath, addition of a drug to the bath, and changing of the bath fluid. If any of these operations be delayed the timing of injections of the drug is likely to be upset and the accuracy of the assay impaired. The present apparatus, which consists of standard component parts of automatic telephone exchanges, has been designed to perform automatically all the operations needed in an assay except the injection of drugs into the bath. Regularity of timing and constancy of fluid volume is thus assured and the operator is left free to concentrate his attention on the solutions to be injected. The method, which has been in use in this laboratory for several years, is particularly useful for carrying out two or more assays simultaneously.

## PRINCIPLE OF THE METHOD

Telephone relays are converted to compress rubber tubing as shown in Fig 1. When the relays are activated the rubber tubing is decompressed and fluid is allowed to flow. These relays control the emptying and filling and the adjustment of fluid level of an isolated organ bath. They are activated at regular time intervals through a telephone uniselector which makes 12 successive contacts in a cycle. The duration of each contact is usually of 15 seconds, thus producing a cycle of 3 minutes. The selector also controls the movements of the drum and a light signal to time the injection of drugs.

The driving magnets of the uniselector are energized through a clockwork mechanism producing electrical pulses at regular time intervals. Since the wipers of the uniselector move on to the next contact every time it is energized, the duration of each step, and consequently of the whole cycle, may be adjusted at will.



If it is desired to alter the *relative* duration of individual operations, for instance the duration of the wash-out period in relation to the whole cycle, the connections of the selector must be rearranged so as to increase or decrease, as the case may be, the number of steps in a cycle. Since the standard P O unselector has eight rows of 25 contacts, whilst only two rows of 12 contacts are needed for the present apparatus, it is possible not only to increase the number of steps per cycle up to 25, but also to have several alternative arrangements on the same selector by utilizing additional rows of contacts which may be switched into the circuit as required

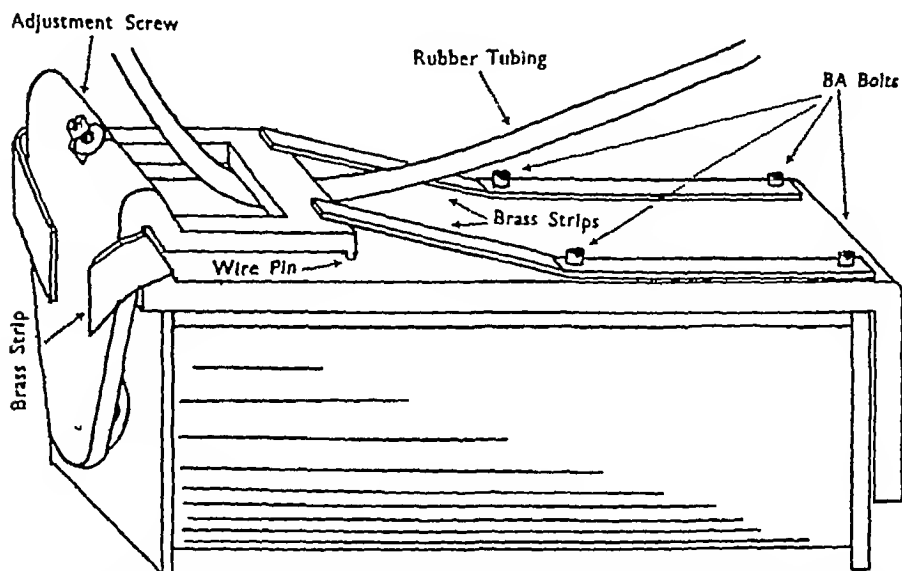


FIG 1—Telephone relay with adjustments enabling it to be used for compressing rubber tubing. Two brass strips screwed in place of the relay contacts act as springs tending to compress the rubber tubing. The strip inserted between relay and armature has the effect of increasing the movement of the armature when the relays are energized

#### DETAILS OF OPERATIONS

The following operations are performed during a  $12 \times 15$  seconds cycle

*Emptying and Filling of the Bath*—The bath fluid is changed twice in succession, the bath being emptied during periods 1 and 3 and filled during periods 2 and 4 by means of the relays shown in Fig 2. A stock bottle containing Tyrode's solution is placed several feet above the table and the rate of inflow is adjusted by means of a screw clip

*Overflow*—The level of fluid in the isolated organ bath is kept constant by means of a bent glass tube fused into its side, which acts as an overflow. Shortly before addition of the drug the bath is allowed to empty to the overflow level through suction from a water pump. During the rest of the cycle the rubber tubing leading to the overflow is compressed by a relay, thus preventing the overflow from acting during the time in which drugs are added to the bath

*Alternative Arrangement for Changing the Bath Fluid*—This would consist in energizing the inflow and overflow relays simultaneously and eliminating the normal outflow altogether. The bath would thus be emptied through the overflow and the tissue would remain continuously immersed in fluid.

*Drum and Signal*—The motor driving the drum is started 15–30 seconds before addition of the drug and is stopped when the bath begins to empty. The

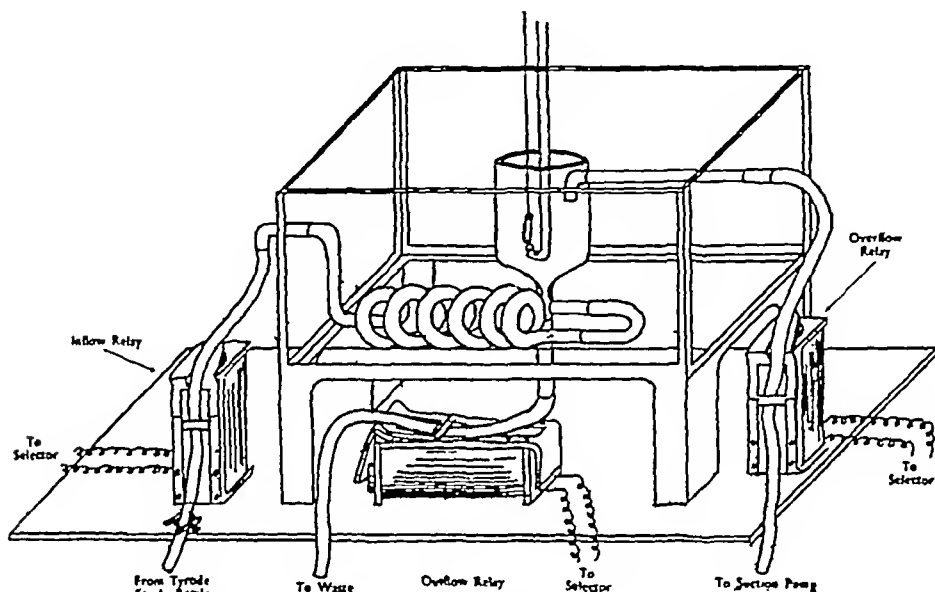


FIG 2.—Bath assembly

light signal for injection of the drug may conveniently be given at the beginning of period 10 or 11, depending on the length of time during which the effect of the drug is to be observed.

#### APPARATUS—DIAGRAM OF CONNECTIONS

The following main pieces of apparatus are required

*Interval Timer*—A "Londex" timer has been used, providing in addition to an interval of 15 seconds, intervals of 6, 10, 15, 20 and 30 seconds.

*Uniselect*—A standard P O uniselect (M.A.I No 1) has been used. It is operated from D.C. mains (230 volts) in series with a suitable lamp resistance. Since the selector has 25 contacts the first and last twelve contacts are wired in parallel whilst the thirteenth contact is jumped by connecting it to the interrupter spring as shown in Fig 3.

*Relays*—Standard P O relays of 3000 ohms resistance are connected directly to D.C. mains without any resistance in series. They thus develop considerable power, whilst owing to the short periods of activation they do not become unduly

hot By means of a few simple adjustments as shown in Fig 1 the gap may be increased sufficiently to take rubber tubing of  $3 \times 5$  mm diameter

*Drum Motor*—If a D.C. motor is available it may be connected directly to the selector without the mediation of a relay as shown in Fig 3

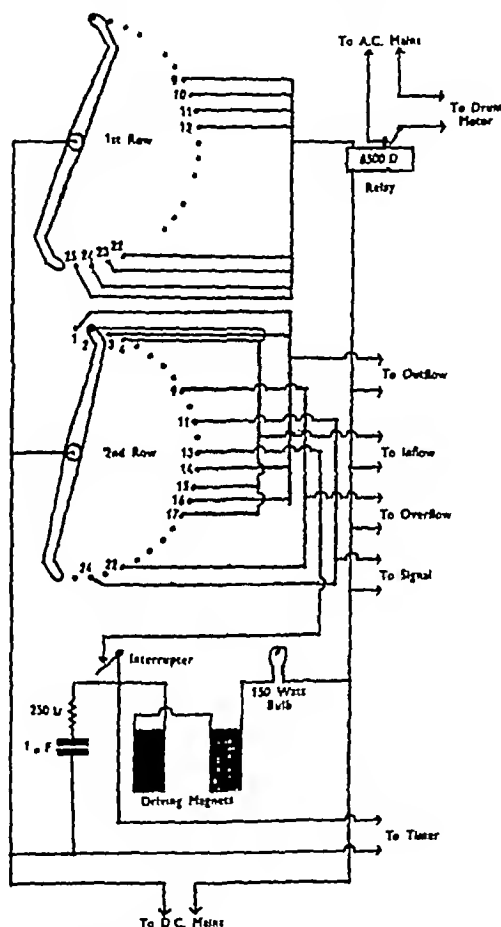


FIG 3—Diagram of connections of unselector

### SUMMARY

Apparatus is described which performs automatically and at regular time intervals all the operations needed in an assay on isolated preparations, save the injection of drugs

### REFERENCE

P O Engineering Dept., Engineering Instructions M.A.I No 1, Unselector

# SULPHONAMIDES IN THE TREATMENT OF CAECAL COCCIDIOSIS OF CHICKENS

BY

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(Received April 3 1946)

Beneficial results obtained with sulphamezathine (sulphadimethylpyrimidine) and sulphadiazine (sulphapyrimidine) in the treatment of caecal coccidiosis in chickens have been reported by Horton-Smith and Taylor (1942, 1943, 1945), who found the mortality among treated chicks to be reduced by 50 to 73 per cent of that among untreated controls in induced epidemics. Hawkins (1943) also obtained satisfactory results when a saturated sulphamezathine solution was substituted for drinking-water 98 hours after infection of chicks. Ripsom and Herrick (1945) found sulphadiazine to be effective when administered in the food, and Swales (1944) found that both sulphamezathine and sulphamerazine (sulphamethylpyrimidine) had a definite curative effect upon established infections even up to the time when intestinal haemorrhage appeared.

Although good results were obtained in the treatment of caecal coccidiosis in chickens by dosing with sulphamezathine incorporated in the food and as a saturated solution in drinking-water, neither of these methods of dosing was perfect. It is difficult in practice to obtain a completely uniform mixture of a small amount of drug with dry food. The low solubility of the drug makes the preparation of saturated solutions of sulphamezathine from the powder troublesome, and such solutions are apt to vary in strength according to the hardness or softness of the water used and the method of preparing the solution. Sulphapyrazine and all the sulphapyrimidine derivatives are more soluble in hard water than in distilled or soft water because the calcium salts are more soluble than the free drugs. Thus a saturated solution in distilled water contained 0.06 per cent sulphamezathine, while a similar solution made in Weybridge tap water at the same time contained 0.13 per cent.

## EXPERIMENTAL

### 1 *The Sodium Salt of Sulphamezathine as a Convenient and Effective Means of Administering the Drug*

1 *Method of Preparation*—The readily soluble sodium salts of sulphonamides can be prepared by dissolving the drugs in a little over the theoretical amount of sodium hydroxide solution or sodium carbonate solution. If sodium carbonate is used the solution must be boiled. A concentrated stock solution of sodium sulphamezathine was prepared

by dissolving 160 g of sulphamezathine in 200 ml of 3N NaOH (12 per cent) and diluting to one litre. Such a solution is marketed by Imperial Chemical (Pharmaceuticals) Ltd. The solution was diluted, just before use, to the concentration required. With hard waters the diluted material slowly produces a deposit of calcium carbonate on exposure to air. An additional advantage of the sodium salts is that one equivalent of alkali is given with the drug, so that the sulphonamides and their acetyl derivatives are unlikely to be deposited in the kidney.

2 *Therapeutic Experiments with Sodium Sulphamezathine after Artificial Infection*—The satisfactory action of sodium sulphamezathine was demonstrated in the following experiments. In each of four experiments one-week-old Light Sussex × Rhode Island Red cockerels were infected by one administration into the crops by pipette of equal heavy infective doses of sporulated oocysts of *Eimeria tenella*. Groups of chicks were given access to different concentrations of sodium sulphamezathine at intervals of 24, 48, 72 and 96 hours after infection. Treatment, which consisted of substituting the solutions to be tested for the ordinary drinking-water, was carried on for approximately seven days after the death of all the controls. The percentage mortality from acute caecal coccidiosis is shown in Table I.

TABLE I

PERCENTAGE MORTALITY FROM CAECAL COCCIDIOSIS AMONG GROUPS OF 50 CHICKS AFTER DELAYED TREATMENT WITH VARIOUS STRENGTHS OF SODIUM SULPHAMEZATHINE SOLUTION

Time that treatment was delayed after infection (in hours)	Percentage mortality at various strengths of sodium sulphamezathine solution, and in control groups.					
	Water control	0.025 per cent	0.05 per cent	0.1 per cent	0.15 per cent	0.2 per cent
24	82	40	14	0	not tested	0
48	100	not tested	14	0	0	0
72	90	not tested	46	26	10	0
96	100	not tested	76	not tested	not tested	50

The surviving chicks were in good condition when they were killed 16 days after the deaths of the last controls. Post mortem examination revealed scattered lesions of coccidiosis in the caeca of chicks receiving the 0.025, 0.05, 0.1 and 0.15 per cent solutions at all times after infection and in all groups treated from the ninety-sixth hour after infection. The experiments point to the 0.2 per cent solution as being the most effective and reliable in controlling the disease. (Percentage strength of all solutions is in grams per 100 ml.)

3 *Sodium Sulphamezathine in the Control of an Induced Epidemic*—A single experiment was carried out with a view to testing the efficacy of three strengths of sodium sulphamezathine solution in controlling such an epidemic as might occur in the field.

Sixty-eight three-week-old chicks were placed on sawdust litter in each of four pens. The litter of each pen was infected with equal quantities of a heavy suspension of sporulated oocysts of *Eimeria tenella*. Blood appeared in the faeces of the chicks of each pen five days after the infection of the litter, the chicks were then randomized by transferring 17 chicks (i.e., a quarter of the number in each pen) to each of the other three pens. Each pen then contained equal numbers of chicks made up of 17 chicks originally present plus 17 from each of the other three pens. This procedure was adopted in an attempt

to correct differences in the original distribution of the oocysts and therefore in infections of the chicks. The chicks in three pens were then given 0.05, 0.1 and 0.2 per cent solutions of sodium sulphamezathine respectively in the place of drinking water. The chicks in the fourth pen served as controls and continued to receive ordinary drinking-water. The results of this experiment are summarized in Table II.

TABLE II

CONTROL OF AN INDUCED EPIDEMIC OF CAECAL COCCIDIOSIS PRODUCED BY *Eimeria tenella* IN GROUPS OF 68 CHICKS BY THE SUBSTITUTION OF SODIUM SULPHAMEZATHINE SOLUTION OF DIFFERENT STRENGTHS FOR THE DRINKING-WATER

No of days after commencement of treatment.	Deaths from caecal coccidiosis among chicks on various percentages of sodium sulphamezathine solution and in a control group			
	0.05 per cent	0.1 per cent	0.2 per cent	Water
0	5	2	3	3
1	3	3	-	7
2	2	-	-	5
3	-	-	-	3
4	-	-	2	2
5	-	-	1	1
6	4	-	-	2
7	3	3	-	11
8	7	2	-	14
9	4	2	-	3
10	-	-	-	3
11	-	-	-	1
12	-	-	-	1
13	-	-	-	-
14	-	-	-	-
Total number of deaths	28	12	6	56

The results compare favourably with those previously reported by Horton-Smith and Taylor (1945) for sulphamezathine, and again show the superiority of the 0.2 per cent solution.

4 *Duration of Treatment with Sodium Sulphamezathine*—An experiment was carried out to determine the minimum time of treatment necessary for effective results. Four groups of 13 chicks each were heavily infected with sporulated oocysts of *Eimeria tenella*. Three groups were given 0.2 per cent sodium sulphamezathine 48 hours after being infected. Ordinary drinking-water was substituted for the solution 24, 48 and 72 hours respectively after the deaths of the fourth group of untreated controls which succumbed to acute caecal coccidiosis on the fifth day. Two deaths from coccidiosis occurred in the 24-hour group and one in the 48-hour group. No deaths occurred in the group that was returned to water three days after the deaths of the controls. The surviving chicks were killed 16 days after the deaths of the controls, and post-mortem findings showed them to be normal apart from some minor lesions in a few of the caeca. From these results it would appear that a treatment carried on for three days after the last deaths from coccidiosis is probably sufficient to control an outbreak of the disease.

Recent work (Asplin, Boyland, and Horton-Smith, 1946) has shown that there are dangers in using sulphamezathine over long periods. If young chicks receive sulphamezathine for two or more weeks the blood-clotting time is lengthened, possibly owing to the decreased synthesis of vitamin K in the gut. In a few cases multiple petechial

haemorrhages of the intestines have been found post mortem after prolonged dosing. Dosing with sulphapyrimidines, particularly sulphamezathine, causes hyperplasia of the semiferous tubules of the testes of cockerels. The testicular enlargement is accompanied by the precocious development of the comb and wattles. It is recommended that the duration of treatment with sulphamezathine should not exceed one week.

## II Comparison of Various Sulphonamides with Sodium Sulphamezathine in the Treatment of Caecal Coccidiosis

1 *Method Used in Tests*—The therapeutic effects of solutions of sulphaguanidine and of the sodium salts of sulphadiazine, sulphamethylpyrimidine, sulphapyrazine, and sulphathiazole were compared with those obtained with solutions of sodium sulphamezathine as substitutes for drinking water. Equal heavy infective doses of sporulated oocysts of *Eimeria tenella* were administered to groups of chicks which later were given the different drugs at different concentrations. One group, the control, remained on drinking-water. In each case one group was treated with sodium sulphamezathine and served as a standard for comparison. The results of these trials are shown in Table III.

2 *Tests made with Sulphapyridine and Sulphaguanidine*—A test was carried out with a ration to which 1 per cent sulphapyridine was added. Groups of chicks had access to the medicated ration 48 hours before and 48 hours after being heavily infected with sporulated oocysts of *Eimeria tenella*. The treatment did not prevent deaths from coccidiosis, 9 of the 10 chicks in each of the treated groups and in the untreated control group succumbed.

Levine (1941), Farr and Allen (1942), and Horton-Smith (1942) have shown that chicks receiving a ration containing 1 to 2 per cent of sulphaguanidine are protected against infection with caecal coccidiosis provided treatment is instituted before the ingestion of the infective dose of oocysts. In view of these findings an experiment was carried out with sulphaguanidine incorporated in the ration on lines similar to those described in Section I for sodium sulphamezathine. Fifty-four chickens were placed in a single pen and the litter was infected. Thirteen days later 10 chickens died of acute caecal coccidiosis. The remaining 44 chickens were then distributed in two groups of 22 chickens each. One group was treated with 2 per cent sulphaguanidine in the food and the other continued to receive a normal ration. Treatment exerted little or no effect on the course of the infection, as 20 of the treated and 21 of the untreated chickens succumbed to the disease.

3 *Resistance of Chickens to Caecal Coccidiosis after Recovery due to Treatment with the Sodium Salts of Sulphamezathine and Sulphapyrazine*—Previous work (Horton-Smith and Taylor, 1945) showed that a strong immunity to coccidiosis developed in chicks which had survived a previous epidemic as a result of treatment. A single experiment was carried out to find whether similar results were obtained when solutions of sodium sulphamezathine and sodium sulphapyrazine were used in treatment.

Three heavily infected groups of 10 chicks each were treated with 0.05, 0.1, and 0.2 per cent sodium sulphamezathine respectively. Another group of 10 chicks was treated with 0.1 per cent sodium sulphapyrazine solution. Treatment was commenced 24 hours after the chicks had been infected. A fifth group received a similar infection, but was maintained on water and served as a control group for the first part of the experiment. A sixth group was not infected, maintained on water, and served as a control for the second part of the experiment. Only one of the chicks from the 0.05 per cent sodium sulphamezathine group died from acute caecal coccidiosis as compared with 10 chicks from the control group. All surviving chicks were restored to water five days after the deaths of the controls. Five days later all the chicks, together with the second control group,

TABLE III

COMPARISON OF SODIUM SULPHAMEZATHINE AND OTHER SULPHONAMIDES INTRODUCED INTO THE DRINKING-WATER IN THE TREATMENT OF CAECAL COCCIDIOSIS.

Test substance (as Na-Salt, except Sulphaguanidine)	Time Treatment was delayed after Administration of Oocysts (in hrs)	Percentage Strength of solution of Test Substance	Mortality after Treatment with Test Substance	Control Groups			
				Mortality after Treatment with Sodium Sulphamezathine of Various Strengths	Mortality among Untreated Control Chicks		
Sulphadiazine (a)	24	0.025	13/16	025	05	01	02
	24	0.05 0.10	13/16 0/10	11/16	3/16	-	-
(b)	24	0.05 0.10	1/8 2/7	2/7	0/7	-	-
Sulphathiazole	24	0.10 0.30	10/10 7/10	-	-	0/10	0/10
Sulphamethyl- pyrimidine (Sulphamerazine)	24	0.05 0.1 0.2	9/13 8/13 0/13	2/13	0/13	0/13	-
Sulphapyrazine	24 (1)	0.025 0.05 0.1	1/7 0/7 0/7	2/7	1/7	0/7	-
	24 (2)	0.1 0.2	0/10 0/10	4/10	1/10	0/10	-
	48	0.025 0.05 0.1	1/6 1/6 0/6	6/6	0/6	0/6	-
	72 (1)	0.2	0/10	4/10	1/10	0/10	-
	72 (2)	0.025 0.05 0.1	2/7 2/7 0/7	6/7	3/7	3/7	-
	24	0.1	4/8	-	-	-	-
Sulphaguanidine	48	0.1	10/16	-	-	-	-
	72	0.1	14/16	-	-	6/16	4/16



received heavy doses of sporulated oocysts. The results of this experiment are set out in Table IV.

TABLE IV

RESISTANCE OF CHICKS, WHICH HAD SURVIVED INFECTION AS A RESULT OF TREATMENT WITH SODIUM SULPHAMEZATHINE AND SODIUM SULPHAPYRAZINE, TO A SECOND HEAVY DOSE OF OOCYSTS ADMINISTERED FIVE DAYS AFTER THEIR RETURN TO WATER

Solution or Water	No of Chicks in Group	Deaths during Treatment	Deaths from Infection after 5 days on Water
Sulphamezathine 0.05%	10	1	1
" " 0.1%	10	0	1
" " 0.2%	10	0	1
Water (1st controls)	10	10	—
Water (2nd controls)*	8	—	7
Sulphapyrazine 0.1%	10	0	3
Water (1st controls)	10	10	—
Water (2nd controls)*	10	—	9

\*The second controls remained uninfected until the chicks which survived the first infection as a result of treatment had received their second heavy infection of oocysts.

4 *Discussion of Results of the Comparisons Made*—Sulphapyridine, sulphathiazole, and sulphaguanidine were all ineffective in treatment of established infections. Sulphadiazine and sulphamethylpyrimidine are both less effective than sulphamezathine. Thus in Table III it will be seen that the mortality was similar in groups treated with 0.1 per cent sulphadiazine and 0.025 per cent sulphamezathine. Similarly, the concentration of sulphamethylpyrimidine required for complete protection (0.2 per cent) was much higher than that of sulphamezathine (0.05 per cent) in groups of chicks dosed at the same time. These results suggest that sulphadiazine and sulphamethylpyrimidine have only about one-quarter of the therapeutic effect of sulphamezathine. The results obtained with sulphadiazine were rather erratic.

The results were interesting in that they showed one sulphonamide, sulphapyrazine, to be more effective than sulphamezathine. In comparative experiments 0.1 per cent sulphapyrazine and 0.2 per cent sulphamezathine have prevented symptoms in almost all chicks even when treatment has been delayed to 72 hours after infection. In practice it is recommended that infections should be treated by the substitution of 0.2 per cent sodium sulphamezathine or of 0.1 per cent sodium sulphapyrazine for the drinking-water as soon as coccidiosis is diagnosed. The work of Asplin, Boyland, and Horton-Smith (1946) has shown that sulphapyrazine and sulphathiazole have no ill effect on the clotting power of the blood or on the testes comparable with that of sulphamezathine.

### III Concentration of the Drugs in the Blood

In an endeavour to gather some information on the mode of action of sulphonamides on the parasite within the epithelial cells we made a study of the concentrations of the various drugs in the blood of chickens of different ages and at various times of day (Tables V and VI).

The concentrations of the sulphonamides in the blood of chickens were estimated by a modification of the method described by Bratten and Marshall (1939). Blood was taken

from the wing vein of adult birds and from the hearts of recently killed young chicks. The blood (0.5 ml) was allowed to haemolyse in 6.5 ml of distilled water for fifteen minutes, after which 1.0 ml of 30-per-cent trichloroacetic acid was added and the precipitated proteins were removed by filtration. The amount of free drug was estimated by comparison of the colour produced in 2 ml of the filtrate after the addition of 0.2 ml of 0.1 per cent sodium nitrite, 0.2 ml of 0.5 per cent ammonium sulphamate, and finally 0.2 ml of 0.1 per cent N-(1-naphthyl)-ethylene diamine hydrochloride, with the colour developed by similar treatment of standard solutions. The amount of total drug was estimated by the same procedure after heating 2 ml. of the blood filtrate with 0.2 ml of 2N hydrochloric acid at 100° C for twenty minutes. The free drug refers to that which is estimated directly and is probably present as such in the blood. The 'total' figures refer to the amount estimated after hydrolysis, and include the drug which is acetylated or in other combined forms.

The individual variations in concentration of sulphamezathine in the blood of groups of chickens (Table V) are considerable, but the differences between

TABLE V

CONCENTRATIONS OF SULPHAMEZATHINE AND SULPHADIAZINE IN MG PER 100 ML. OF BLOOD OF CHICKENS RECEIVING DIFFERENT STRENGTHS OF THESE DRUGS IN WINTER

Drug	Times Samples were Taken	Concentration of Drug in the Drinking water								
		0.05 per cent			0.1 per cent			0.2 per cent		
		Free Drug	Total Drug	Percentage of Total Present as Free Drug	Free Drug	Total Drug	Percentage of Total Present as Free Drug	Free Drug	Total Drug	Percentage of Total Present as Free Drug
Sulphamezathine	7.30 a.m.	1.4	2.0	70	4.9	6.3	78	8.5	9.9	86
		2.5	3.2	78	3.3	4.3	77	5.7	5.8	98
		1.9	2.4	79	3.9	5.2	75	6.3	8.0	79
		1.6	2.5	64	2.3	2.5	92	5.5	7.1	78
Mean		1.8	2.5	73	3.6	4.6	80	6.5	7.7	85
	3.30 p.m.	3.6	6.1	59	9.1	9.9	93	17.9	19.6	91
		2.0	2.2	91	5.7	5.8	98	10.0	12.0	83
		2.7	4.4	62	4.3	6.6	65	15.8	17.3	91
		2.5	2.9	86	4.9	5.1	96	12.2	14.6	83
Mean		2.7	3.9	75	6.0	6.8	88	14.0	15.9	87
Sulphadiazine	Morning	1.5	2.7	56	1.6	1.7	94	10.3	11.1	93
		1.4	2.5	56	3.8	5.0	76	3.0	3.1	97
		2.6	2.7	96	1.5	3.2	47	0.5	0.6	83
		3.1	3.4	91	7.3	8.1	90	0.4	0.7	57
Mean		2.1	2.8	75	3.5	4.5	76	3.5	3.8	82
	Evening	4.9	5.9	83	1.4	2.4	58	1.3	2.4	54
		5.4	6.7	81	1.3	2.6	50	12.6	15.9	79
		3.7	3.9	95	9.5	9.8	97	14.1	4.7	96
		4.1	4.6	89	6.7	8.5	79	6.1	6.3	97
Mean		4.5	5.3	87	4.7	5.9	71	8.3	9.8	82

different levels of dosing and between morning and evening are quite clear and greatly exceed individual variation. The concentrations of sulphadiazine showed much individual variation except when a low level (0.05 per cent in drinking-water) was administered. There was also considerable variation in curative effect with sulphadiazine, the two effects may be related and be due to some individual chickens not drinking or not absorbing the more concentrated sulphadiazine solutions. The blood concentrations given in subsequent tables and Figure 1 are

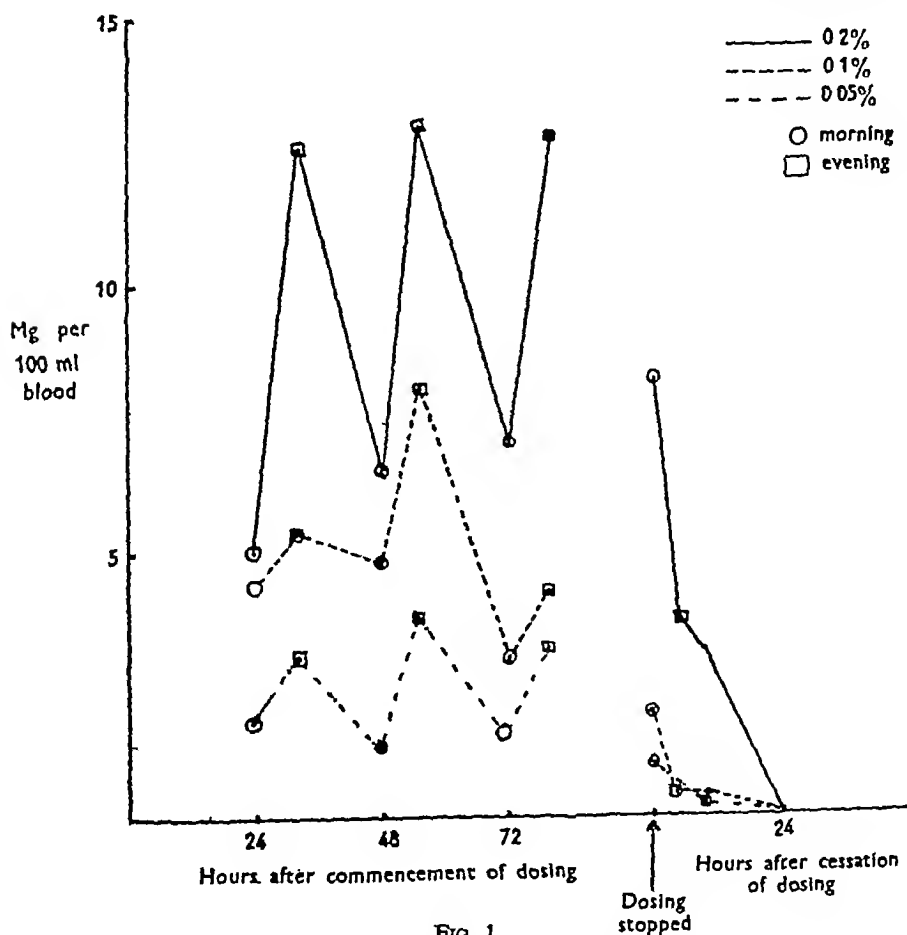


FIG 1

all mean values for four chickens sampled at the same time. The individual variations in blood concentrations in the case of the other drugs examined were much less than with sulphadiazine. The variation in concentration of sulphamezathine with time of day and the fall in concentration after cessation of dosing are shown in Table V (one-week old chicks) and Figure 1 (twelve-week old chickens).

TABLE VI

CONCENTRATIONS OF SULPHAMEZATHINE, SULPHAPYRAZINE AND SULPHATHIAZOLE (IN MG PER 100 ML.) IN THE BLOOD OF CHICKS IN WINTER AND SUMMER. CONCENTRATIONS WERE DETERMINED AT LEAST 48 HOURS AFTER COMMENCEMENT OF DOSING AND ARE MEAN VALUES FOR FOUR CHICKS SAMPLED AT THE SAME TIME.

Drug and Season When Dosed		Concentration of Drug in Drinking Water									
		0.05%			0.10%			0.20%			
		Free Drug	Total Drug	Percentage of Total Present as Free Drug	Free Drug	Total Drug	Percentage of Total Present as Free Drug	Free Drug	Total Drug	Percentage of Total Present as Free Drug	
Sulphamezathine	Winter	One week old	Morning Evening	18 30	24 38	75 79	37 58	52 94	71 62	97 130	73 78
		Two weeks old	Morning Evening	20 31	28 42	72 72	36 52	50 79	72 66	139 131	67 71
		Twelve weeks old	Morning Evening	14 34	23 52	61 65	39 57	56 84	70 68	78 144	85 72
	Summer	Two weeks old	Morning Evening	39 33	42 40	93 83	80 86	102 110	78 78	119 126	91 83
Twelve weeks old		Morning Evening	19 20	28 30	68 66	51 61	55 64	93 95	102 102	90 90	
Sulphapyrazine	Summer	Two weeks old	Morning Evening	57 39	64 45	89 87	87 60	94 66	92 91	179 156	94 92
		Twelve weeks old	Morning Evening	17 27	20 36	85 75	37 42	43 48	86 87	113 120	88 86
Sulphathiazole	Summer	Two weeks old	Morning Evening	10 11	11 15	91 73	0.9 0.9	0.9 1.4	100 65	26 15	92 60

When the drug was administered as a 0.2 per cent solution the blood concentration was very rarely below 5 mg per 100 ml. As the most satisfactory therapeutic results were obtained by dosing with the 0.2 per cent solution it appears that a blood concentration of 5 to 10 mg. per 100 ml is necessary for optimal results. There was a tendency for the blood concentrations to be higher in younger chicks when dosed in summer. The blood concentrations are of the order of one-twentieth of the concentration of drug in the drinking-water. Table VI indicates the mean values of concentrations obtained in chicks of different ages in winter and summer. The blood concentrations varied with the concentrations of drug in the drinking-water and to some extent with the time of day. Samples of blood taken at 7.30 a.m. (G.M.T.) in the winter had consistently lower concentrations than similar samples taken at 3.30 p.m. (G.M.T.). This is presumably due to the chicks drinking less during the hours of darkness, so that the blood concentrations fall during the night. Similar determinations carried out in the summer (June) showed no regular variations between morning (6.30 a.m., G.M.T.) and afternoon (2.30 p.m., G.M.T.) values.

The concentrations of sulphapyrazine in young chicks (two weeks old) were considerably higher than in older (twelve weeks old) chickens treated at the same time. The difference with age is similar but much greater than that noticed with sulphamezathine. The concentrations of sulphapyrazine were higher than those for comparable dosage levels of sulphamezathine and the higher therapeutic action is probably due to the higher blood concentrations obtained. It is remarkable that sulphapyrazine should be so well absorbed when it is poorly absorbed from the alimentary tract of mammals (cf. Robinson *et al.*, 1943).

The differences in blood concentrations obtained in chickens and canaries after oral administration of sulphonamides have been described by Marshall (1945). In his experiments sulphapyridine derivatives appeared to be more easily absorbed and more slowly excreted than was sulphathiazole, and the antimalarial activity was correlated with the attainable blood concentration. In our experiments the values for sulphathiazole, which are given in Table VI, show that this drug is poorly absorbed or quickly excreted, and possibly has no therapeutic action for this reason.

Sulphapyridine, given as 1 per cent of the chickens' ration, was fairly well absorbed. The blood concentrations were as follows: morning values were, free 6.2 and total 6.6 mg per 100 ml, while evening values were, free 6.5 and total 7.6 mg per 100 ml. Sulphapyridine is ineffective in curing the disease presumably because it does not inhibit the growth of coccidia.

Sulphaguanidine is relatively poorly absorbed from the alimentary tract of mammals and is therefore used in the treatment of intestinal diseases. When a solution (0.1 per cent) of this drug was given to chicks the blood concentrations appeared to rise very slowly (Table VII). Even so, the concentrations obtained after five days were insufficient to have a curative or prophylactic effect as experiments have shown. When 1 to 2 per cent of sulphaguanidine was administered in

the food it was found to have a prophylactic effect, and under these conditions the blood concentrations were quite high and appeared to be rapidly attained. The method of determination would not differentiate between sulphaguanidine and any derivative (with an intact aminophenyl group) which might be formed from sulphaguanidine either in the gut or in the body.

TABLE VII

CONCENTRATION OF SULPHAGUANIDINE IN THE BLOOD OF CHICKS AT DIFFERENT TIMES FOLLOWING THE COMMENCEMENT OF DOSING

Concentration of Drug and the Means of Dosing	Time after Commencement of Dosing in Hours	Sulphaguanidine mgs. per 100 ml. Blood	
		Free	Total
0.1% in drinking-water	16	1.8	2.1
	26	1.9	2.6
	42	2.2	2.5
	66	2.6	3.2
	84	3.3	4.5
	108	3.2	3.7
	192	4.0	4.4
1% in food	24	7.6	8.1
	96	5.7	7.5
	120	5.3	6.1
2% in food	24	20.0	21.8
	96	20.9	24.6
	120	19.5	27.3

#### IV Antagonism of Sulphonamide Action by *p*-Aminobenzoic Acid

The bacteriostatic action of sulphonamides on streptococci has been shown to be inhibited *in vitro* by *p*-aminobenzoic acid (Woods, 1940), and in view of experiments on the neutralization of the therapeutic action of sulphonamides in small animals (Selbie, 1940) it would seem probable that a similar mechanism would operate *in vivo*. An experiment was carried out to test this assumption in the case of avian coccidiosis. Groups of chicks, heavily infected with coccidia, were given (1) sulphamezathine and sulphapyrazine solutions, (2) these solutions with the addition of different concentrations of *p*-aminobenzoic acid (PAB), and (3) PAB only. As *Eimeria tenella* cannot be grown *in vitro* the effect can only be shown in infected chicks.

The results (Table VIII) show that the therapeutic effect of 0.2 per cent sulphamezathine was largely neutralized by the presence of 0.01 per cent PAB and that of 0.1 per cent sulphamezathine by 0.005 per cent PAB. This means that the therapeutic action of ten molecules is neutralized by one molecule of PAB. The action of 0.05 per cent sulphapyrazine was neutralized by 0.002 per cent PAB. Compared with the effect of sulphanilamide on streptococci *in vitro*, where the amount of PAB required to neutralize the effect of the drug is small (i.e., one molecule of PAB nullifying the effect of several thousand molecules of the drug), the amount of PAB required to neutralize sulphamezathine or sulphapyrazine is large (i.e., ten molecules are neutralized by one molecule), the ratio

being of the same order as that found in the treatment of streptococcal infections of mice (Selbie, 1940) The action of sulphamezathine in the animal appears to be dependent on the use of PAB by the coccidia

TABLE VIII

THE EFFECT OF DIFFERENT CONCENTRATIONS OF PAB ON THE THERAPEUTIC EFFECT OF SULPHAMEZATHINE AND SULPHAPYRAZINE

Percentage Concentrations of Drugs and of PAB			Mortality
Sulphamezathine	Sulphapyrazine	PAB	
<i>Exp A</i>			
—	—	0.1	6/6
0.2	—	—	0/7
0.2	—	0.1	8/8
0.2	—	0.01	5/8
0.2	—	0.001	1/7
<i>Exp B</i>			
Controls			
—	—	—	10/10
0.1	—	—	0/10
—	—	0.02	10/10
0.1	—	0.02	10/10
0.1	—	0.01	10/10
0.1	—	0.005	6/10
0.1	—	0.002	6/10
—	0.05	—	0/10
—	0.05	0.005	8/10
—	0.05	0.002	9/10

## DISCUSSION

The results show clearly that a protozoal infection can be cured by certain heterocyclic sulphonamides and that the therapeutic action resembles the antibacterial action of sulphonamides in being neutralized by *p*-aminobenzoic acid. The two most effective drugs, sulphamezathine and sulphapyrazine, differ from each other in some properties to a greater extent than does sulphamezathine from the less effective compounds like sulphadiazine and sulphamethyldiazine. Thus sulphapyrazine is much less soluble in neutral solution than the three sulphapyrimidine compounds. The effects of the different drugs are summarized in Table IX.

The difference in the therapeutic efficiencies of the drugs is no doubt partly due to differences in absorption or excretion, thus, with the effective compounds, the therapeutic value increases with the concentration of the drug in the blood for a given dosage level. But absorption (or delay in excretion) is not the only factor, because sulphapyridine is absorbed but has no therapeutic action, it may be assumed that this drug is not toxic to the parasite. Consideration of the similarity of the effects of sulphathiazole and sulphadiazine on the growth of bacteria *in vitro* would suggest that the variation in, or lack of, effect on coccidia

is due to the poor absorption of the drug by the host. In our present state of knowledge of sulphonamide metabolism the unpredictable variations in absorption between different drugs in different species, and in chickens of different ages, can only be found by trial. From the fact that only drugs which are absorbed appear to be effective in the treatment of chickens already infected, and consider-

TABLE IX

EFFECT OF DIFFERENT SULPHONAMIDES ON INFECTIONS OF *Eimeria tenella* IN CHICKENS

Drug	Effect on <i>Eimeria tenella</i> Infection	Number of Hours after Infection that Treatment was Delayed
Sulphanilamide	No prophylactic effect (Levine, 1939)	—
Sulphapyridine	No prophylactic effect although absorbed	—
Sulphathiazole	1-2% was effective when administered in food before or at time of infection (Ripsom and Herrick, 1945). Dosing gives low blood concentrations.	—
Sulphaguanidine	2% in food had prophylactic effect if fed to chickens before infection	—
Sodium Sulphadiazine	0.1% in drinking-water had some therapeutic effect.	24*
Sodium Sulphamerazine	0.2% in drinking-water had some therapeutic effect.	24*
Sodium Sulphamezathine	0.2% in drinking-water had excellent therapeutic effect.	24-72
Sodium Sulphapyrazine	0.1% in drinking-water had excellent therapeutic effect.	24-72

\*Longer delays in treatment were not carried out with these sulphonamides

ing that the life-cycle of the protozoon is principally intracellular, it would seem that effective treatment for caecal coccidiosis consists in attacking the parasite in the tissues.

#### SUMMARY

1 Caecal coccidiosis in chickens caused by the protozoon *Eimeria tenella* can be effectively treated by substituting solutions of 0.2 per cent sodium sulphadimethylpyrimidine (sodium sulphamezathine) or 0.1 per cent sodium sulphapyrazine for drinking-water. Sulphadiazine and sulphamethylpyrimidine (sulphamerazine) will also cure the infection but are not so reliable as the other drugs. Sulphathiazole and sulphapyridine are completely ineffective in preventing symptoms of caecal coccidiosis in infected chicks.

2 The relative therapeutic effectiveness of sulphapyrazine and the sulphapyrimidines depends upon the blood concentrations obtained. Effective doses result in blood concentrations of 5 to 10 mg per 100 ml.



3 Chickens which survive an infection of caecal coccidiosis as a result of treatment with sulphamezathine or sulphapyrazine are resistant to subsequent infections with *Eimeria tenella* within the period tested

4 The action of sulphamezathine and sulphapyrazine on coccidia in the chicken is antagonized by *p*-aminobenzoic acid

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# THE PHARMACOLOGY OF BASIC ESTERS OF THIAZOLE CARBOXYLIC ACIDS

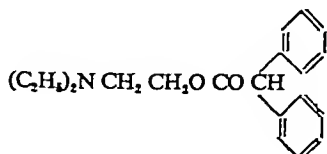
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M R A CHANCE, P DIRNHUBER AND F A ROBINSON

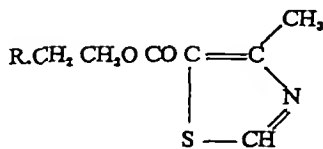
*From Glaxo Laboratories, Ltd Greenford, Middlesex*

(Received March 2 1946)

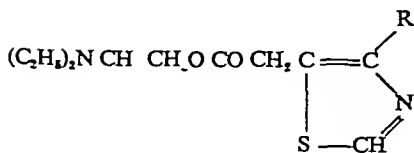
During an investigation into the pharmacological properties of thiazole compounds, several basic esters of 4-methyl-thiazole-5-carboxylic acid (II) and the diethylaminoethyl esters of 4-methyl-thiazole-5-acetic acid (III, R=CH<sub>3</sub>), thiazole-5-acetic acid (III, R=H) and 2-amino-4-methyl-thiazole-5-carboxylic acid (IV) were prepared (Jones, Strachan and Robinson, 1946), these have now been examined pharmacologically. The close chemical relationship between these compounds and Trasentin (I) suggested that they might possess spasmolytic properties, and this has been confirmed *in vitro*. The appearance, moreover, of convulsions produced by the injection of non-toxic doses of these compounds into rabbits, mice, and guinea-pigs revealed stimulant properties on the CNS, which have been studied. Finally, the similarity in structure of diethylaminoethyl-2-amino-4-methylthiazole-5-carboxylate (IV) and procaine prompted us to investigate the local anaesthetic properties of this compound.



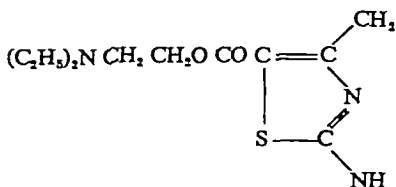
I



II



III



IV

NOTE.—Trasentin-6H is hexahydro-trasentin, i.e., one phenyl group of (I) replaced by cyclohexyl

## EXPERIMENTAL

I *Spasmolytic Properties*

1 *Action on isolated organs*—The normal rhythm of the isolated ileum of the rabbit in Ringer's solution\* was suppressed after a few minutes by a concentration of 1.25 mg./ml diethylaminoethyl 4-methyl-thiazole-5-carboxylate (II,  $R=(C_2H_5)_2N$ ) and was reduced, but not inhibited, by one-fifth of this concentration. In different experiments the tone fell, rose, or remained the same. Recovery took place within a few minutes after removal of the drug. Trasentin-6H, in a concentration of 2.5  $\mu$ g/ml, produced no effect, but 12.5  $\mu$ g/ml, i.e., 1/100 the concentration of the thiazole compound, produced a lowering of the tone with suppression of the rhythm.

The diethylaminoethyl ester, unlike Trasentin which lowers muscle tone, raised the tone of the isolated uterus of the rabbit and guinea pig in Ringer's solution. The effect was similar to that of posterior pituitary extract, except that the amplitude of the spontaneous contractions following this rise in tone was increased by the diethylaminoethyl ester, but remained the same with posterior pituitary extract. The increase of tone produced and maintained by 2 mg of the ester was approximately the same as that produced by 0.05 IU posterior pituitary extract or by 60  $\mu$ g atropine sulphate on guinea pig uterus.

A concentration of 1.25 mg/ml of the diethylaminoethyl ester contracted the bladder muscle of the guinea-pig.

2 *Action on isolated organs treated with acetylcholine (Ach)*—Preliminary tests on the Ach spasm of the isolated rabbit ileum in Ringer's solution showed that the esters listed in Table I had a weak spasmolytic action. Their activities were compared by measuring the degree of relaxation induced in a strip of ileum which was responding to a concentration of 0.4 ppm Ach with constant submaximal contractions. The degree of

TABLE I

*In vitro* ACTIVITY AGAINST ACETYLCHOLINE ( $4 \times 10^{-7}$ ) IN RABBIT ILEUM

Compound ( $10^{-4}$ )	Per cent Relaxation of Contracted Muscle
Piperidinoethyl 4-methylthiazole-5-carboxylate	58
Diethylaminoethyl " "	53
Dimethylaminoethyl " "	50
$\beta$ -Diethylaminopropyl " "	48
Morpholinoethyl " "	33
Diethylaminoethyl 2-methylthiazole-4-acetate	22

relaxation, expressed as the percentage reduction of this contraction, was measured for a standard dose of thiazole compound (see Table I). The piperidinoethyl ester of 4-methyl thiazole-5-carboxylic acid (II,  $R=C_2H_5N$ ) produced the maximum relaxation, followed by the dimethylaminoethyl ester (II,  $R=(CH_3)_2N$ ) and, because the former was also the most active musculotropic compound, its spasmolytic activity was assayed against Trasentin by a method which involved 'bracketing' doses of test and standard spasmolytic, as in the method of Dale for the assay of posterior pituitary extracts. It was found impossible to treat a single piece of gut with a sufficient number of doses of spasmolytic to make a randomized order feasible, this was due to the effects of Trasentin persisting for long periods and interfering with subsequent doses. Tested by this method the piperidinoethyl ester had approximately 1/1,000 of the activity of Trasentin.

\*The bath containing the isolated tissue had a capacity of approximately 40 ml, and this value was used in calculating the concentrations recorded in the text.

A concentration of 0.5 mg/ml of the diethylaminoethyl ester completely suppressed the contractions produced by a concentration of 0.1 p.p.m. Ach whilst 125 mg/ml was required to antagonize the effect of 0.4 p.p.m. Ach. Under the same conditions 5 µg/ml Trasentin was sufficient to suppress the contractions produced by 0.4 p.p.m. Ach, so that Trasentin was apparently 250 times as active. The recovery of the normal rhythm after treatment with Trasentin was proportional to the degree of relaxation induced, whereas the recovery of rhythm by the ileum relaxed by the thiazole compound was not dependent on the degree of relaxation and was very irregular.

B. B. Dikshit (1938) has reported that isolated rabbit ileum kept in Ringer's solution for 98 hours at 1°C is unable to synthesize Ach and he considered this synthesis to be mainly a function of the nerve plexus in the intestinal wall. This may provide a method of inhibiting Auerbach's plexus and so producing a denervated preparation on which the action of plain muscle stimulators and spasmolytic substances can be tested free from interference by the nerve plexus. These preparations are not as sensitive to Ach as is the normal isolated gut but good contractions can be obtained with 10 µg. Ach which is the quantity used throughout this work to produce contractions of the isolated gut. Observations on four strips of ileum from two rabbits showed that the response to 0.1 p.p.m. Ach was completely inhibited by a concentration of 1 mg/ml of the diethylaminoethyl ester, and that the contracted ileum was relaxed to twice its original length by 0.2 mg/ml.

A concentration of 0.2 mg/ml of the dimethylaminoethyl ester also reduced the contraction to 0.1 p.p.m. Ach but to a smaller extent than an equal concentration of the diethyl compound. In two further tests a concentration of 1 mg/ml completely inhibited the action of 0.1 p.p.m. Ach on a strip of ileum from another rabbit. These results suggest that the spasmolytic action is exerted directly on the muscle and is not mediated through Auerbach's plexus.

3 *Action on isolated organs treated with histamine or barium chloride*—The effects of each compound on the spasm produced in the isolated guinea-pig ileum suspended in Tyrode by a concentration of 2 p.p.m. histamine hydrochloride was tested at four different levels and the percentage relaxation plotted against dosage. The resulting graphs proved to be straight lines. The relative activities of the compounds, as set out in Table II, were calculated from the concentrations required to produce half-relaxation. The dimethylaminoethyl ester was the most active member of the series and had one-quarter the activity of Trasentin-6H.

TABLE II

*In vitro* ACTIVITY AGAINST HISTAMINE HYDROCHLORIDE ( $2 \times 10^{-4}$ ) IN GUINEA-PIG ILEUM

Compound	Concentration Producing Half Relaxation
Dimethylaminoethyl 4-methylthiazole-5-carboxylate	$3.8 \times 10^{-5}$
Diethylaminoethyl	$6.0 \times 10^{-5}$
Piperidinoethyl	$7.2 \times 10^{-5}$
β-Diethylaminopropyl	$1.7 \times 10^{-4}$
γ-Diethylaminopropyl	$2.1 \times 10^{-4}$
Morpholinoethyl	$2.7 \times 10^{-4}$
Diethylaminoethyl 2-amino-4-methylthiazole-5-carboxylate	$2.2 \times 10^{-4}$
Diethylaminoethyl 2-methylthiazole-4-acetate	$1.5 \times 10^{-3}$
Diethylaminoethyl thiazole-4-acetate	$1.8 \times 10^{-3}$
Trasentin-6H	$1.0 \times 10^{-2}$

The effects of the compounds on the barium chloride contractions of the isolated guinea-pig ileum resembled those produced on the rabbit ileum except that the compounds

permanently disturbed the normal rhythmical contractions. The barium chloride contractions of the isolated rabbit's ileum were also inhibited.

4 'In vivo' experiments—Attempts to obtain in the living animal the reactions shown by the isolated tissues have failed in every instance except for the heart rate. The movements of a loop of ileum in a rabbit were recorded by a kymograph needle connected to it by a thread. 10 mg. of the diethylaminoethyl ester failed to diminish the violent movements induced by 25 mg.  $\text{BaCl}_2$  injected intravenously. Similar results were obtained in the guinea-pig after intracardial injections. A dose of 2.5 mg. intravenously produced a lowering of the heart rate in three rats, as recorded by the electrocardiograph, but this is probably a result of vagal activity following CNS stimulation (see below), for this dose is close to the convulsive level by the intravenous route.

## II Central Nervous Stimulation

The convulsions produced by the dimethyl- and diethyl-aminoethyl esters of 4-methyl-thiazole-5-carboxylic acid were compared with those produced in rabbits by intravenous injection of leptazol and Trasentin-6H in quantities shown in Table III. The doses quoted in the Table were determined by injecting six rabbits with graded doses starting at the LD 50 and diminishing in size.

TABLE III

MINIMAL CONVULSIVE DOSES BY INTRAVENOUS INJECTIONS TO RABBITS

Substance	Dose (mg./kg.)
Diethylaminoethyl 4-methyl thiazole-5-carboxylate	87.5
Dimethylaminoethyl 4-methyl-thiazole-5-carboxylate	62.5
Leptazol	10.0
Trasentin-6H	15.0

At the onset of a convulsion produced by either thiazole compound, the rabbit sits back on its haunches with its forelegs extended. This takes place within a minute. A clonic phase ensues with violent running motions of the fore and hind legs, and passes into a tonic phase with opisthotonus. At this point the animal loses its balance and becomes unconscious. The tonic phase lasts no longer than one minute and is followed immediately by a return of consciousness and clonic movements which pass off leaving the animal exhausted. During the first clonic phase and part of the tonic phase the respiration is completely inhibited, but as the latter passes off the rate of respiration increases and apparently also the amplitude. Rabbits convulsed with the dimethyl compound always recovered their normal sitting position within ten minutes, usually soon after they recovered consciousness. Convulsions produced by the diethyl compound, on the other hand, were followed by a long period of exhaustion and the animal rarely recovered its balance in less than fifteen minutes, sometimes it took as long as half an hour. These convulsions are identical with those produced by leptazol, except that it is not possible with the thiazole compounds to induce such violent clonic movements involving the whole of the trunk and limbs, and the convulsions do not last as long.

Observations were also made to discover whether any difference existed between the convulsions produced in intact frogs and in frogs in which the higher nervous centres were separated from the spinal cord. It was observed that with intact frogs the thiazole compounds and leptazol produced convulsions similar to those obtained with strychnine, whereas in pithed frogs the effect was less marked and lasted for a much shorter period than with strychnine. The results suggest

that the higher centres as well as the spinal cord are involved in the stimulation produced by the thiazole compounds and by leptazol

J W Schultz, L. M Tainter, and J M Dill (1939) describe a method of distinguishing between cortical and sub-cortical stimulation by measuring the total activity exhibited by rats during a period of seven hours. The rat, which has received a subcutaneous dose of the test substance is suspended in a cage by a spring which oscillates as the animal moves about the cage. By recording the number and extent of the oscillations, an arbitrary measure of the amount of the activity exhibited in unit time can be recorded. The authors distinguish by this method between leptazol and picrotoxin, on the one hand and nikethamide and caffeine on the other the last two substances producing a marked increase in activity which after nikethamide injections extends over a period of six hours and after injection of caffeine over a period of three hours. Leptazol and picrotoxin, on the other hand, only produce a brief period of activity extending to not more than one hour, and this may be partly due to convulsions. We have made similar observations with the thiazole compounds and amphetamine. The thiazole compounds did not appear to increase the total activity but, indeed, appeared to depress it soon after the injection. It may, therefore, be concluded from the results reported in these three sections that central nervous stimulation by these compounds is primarily a medullary stimulation with involvement of the spinal cord.

### ANALEPTIC ACTIVITY

The results described in the previous three sections suggested that the thiazole compounds might antagonize the action of anaesthetics on the central nervous system. Tests were therefore made to ascertain their effect on (a) the duration of anaesthesia, and (b) the toxicity of anaesthetics. Two different types of anaesthetics were chosen for the main investigations, namely, amytal sodium as a representative of the barbiturates, and paraldehyde which belongs to a different group of compounds, but produces anaesthesia of approximately the same duration.

#### (a) *Reduction of Anaesthetic Time*

From the dosage-mortality curve of amytal it was ascertained that no significant mortality would be expected in groups of animals receiving a dose equal to 60 per cent of the LD 50, as this dose produced a satisfactory period of anaesthesia, it was selected to produce the standard degree of anaesthesia. The same proportion of the LD 50 of paraldehyde was also found to be satisfactory.

Two analeptic drugs differing in their action on the nervous system were chosen for comparison with the two thiazole compounds, namely, picrotoxin and  $\beta$ -phenylisopropylamine sulphate (amphetamine). Picrotoxin is used as an antidote in barbiturate poisoning because it is safe in doses far exceeding those lethal to unanaesthetized animals although its action is relatively short and somewhat irregular. Amphetamine, on the other hand, although as toxic to unanaesthetized as to anaesthetized animals has a more prolonged action than picrotoxin (compared by the percentage recovery of mice) and is more effective at dose levels proportionately further removed from the LD 50 for unanaesthetized animals. It was, however, found impossible to obtain even an approximate value for the toxicity of amphetamine, and a search of the literature showed that other workers had experienced the same difficulty as much as a tenfold difference is reported in the figures quoted by different authors using the same route for mice. It was therefore considered necessary to make an exhaustive study of the matter the results of which finally enabled

us (Chance, 1946), after the conditions of the test had been sufficiently defined and controlled, to determine the toxicity of this substance with the same degree of accuracy as is usually encountered in biological assays. Meanwhile, a comparison was made of the two substances already mentioned with picrotoxin. Groups of sixteen mice received the standard dose of 120 mg./kg. by intraperitoneal injection followed immediately by a subcutaneous injection of the anaesthetic.

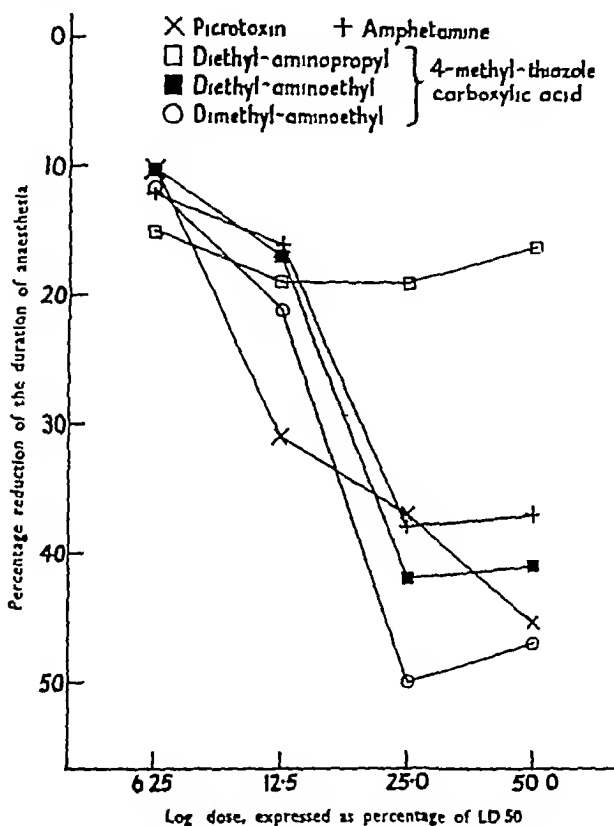


FIG 1—Shortened duration of amylal anaesthesia

Both thiazole compounds, like picrotoxin, reduced the duration of anaesthesia produced by the standard dose of amylal (Fig 1). The reductions in the duration of anaesthesia, brought about by equivalent proportions of the LD<sub>50</sub> of the two thiazole compounds and picrotoxin, were approximately equal, when the logarithm of the dose was plotted against the reduction in anaesthetic time the relationship was not linear for the thiazole compounds, though possibly so for picrotoxin.

The thiazole compounds were, however, less effective than picrotoxin against paraldehyde, and acted somewhat differently (Fig 2). Over the same range of doses, expressed as proportions of the LD<sub>50</sub> the dimethyl compound was less

effective than the diethyl compound which had an optimal activity at a level equal to 25 per cent of its LD 50. These compounds are clearly less active than picrotoxin but their action is nevertheless pronounced, and against amytal their action is indistinguishable from that of picrotoxin when compared at the same proportions of their respective LD 50's

After the investigation into the factors affecting the toxicity of amphetamine had been completed, two more compounds became available. These were the hydrochlorides of diethylaminopropyl 4-methyl-thiazole-5-carboxylate

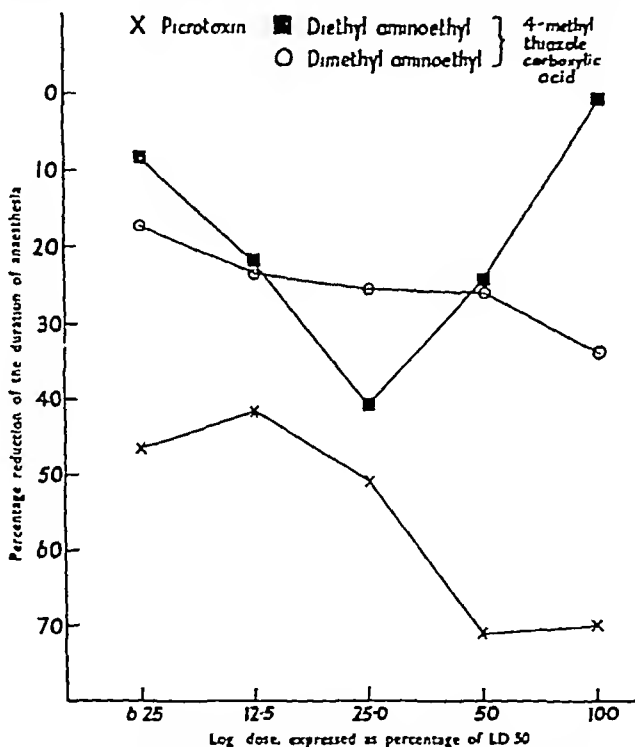


FIG 2.—Shortened duration of paraldehyde anaesthesia

(II, R =  $(C_2H_5)_2NCH_2$ ) and diethylaminoethyl 2-amino-4-methyl-thiazole-5-carboxylate (IV). The former produced convulsions on injection into mice, and was therefore tested for analeptic activity, together with the hydrochlorides of the diethyl- and dimethylaminoethyl esters of 4-thiazole-5-carboxylic acid. On this occasion amphetamine sulphate was also included in the comparison. The combined results are shown in Fig 1.

#### (b) Effect on the Toxicity of Amytal

The LD 50 of amytal sodium on "Swiss" mice had been measured previously and found to be 200 mg per kg. body weight. When redetermined on a group of twenty mice it appeared to be slightly higher, and it is possible that the original estimate may have been



somewhat low. A dose of 200 mg/kg was, however, chosen as the toxic dose in these experiments, and gave a mortality of 43 per cent.

The values of LD 50 for the diethyl and dimethyl compounds are 700 and 500 mg. per kg. respectively, for strychnine 1 mg. per kg. and for picrotoxin 5 mg. per kg. Each substance was tested by subcutaneous injection into a group of not more than ten mice immediately after intraperitoneal injection of the standard dose of amytal. Both the amytal and the various amounts of the test substances were administered in equal volumes of solution. The procedure was completed in a total of four minutes, so that each received the antidote at the onset of anaesthesia, which for amytal occurs two minutes after intraperitoneal injection. In each investigation carried out on one day the same number of animals was used for each dose of all four substances, and simultaneously the same number of animals received the standard dose of amytal alone. The whole investigation was then repeated until all doses of each substance had been tested on forty animals.

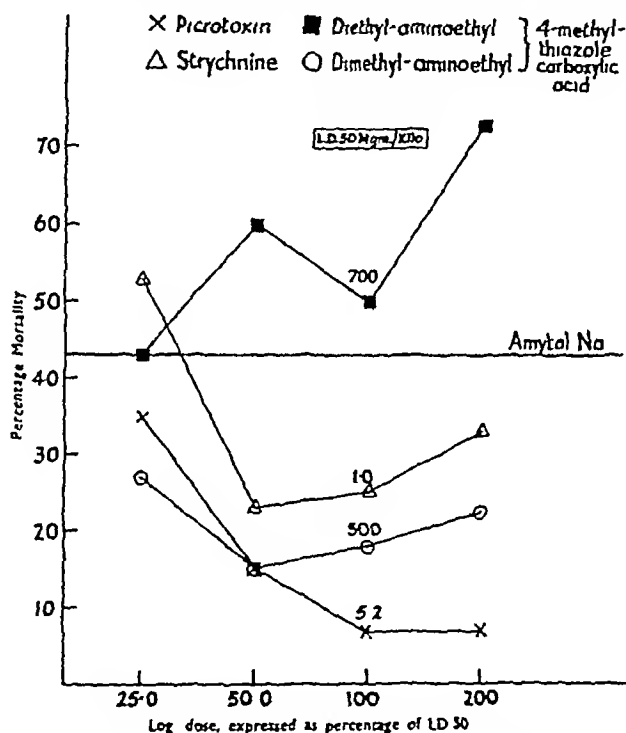


FIG. 3.—Effect of picrotoxin, strychnine and the dimethyl- and diethylaminoethyl esters of 4-methyl-thiazole carboxylic acid on the toxicity of amytal sodium (200 mg./kg.) in mice.

Fig. 3, in which the dose, expressed as the logarithm of the percentage of the LD 50, is plotted against the percentage mortality for each substance, shows that the mortality to the standard dose of amytal was 43 per cent—a slightly lower value than expected. Reductions in mortality are statistically significant when the mortality was 20 per cent or less. The three highest doses of picrotoxin produced a significant lowering of mortality from the standard dose of amytal, though the lowest did not. With strychnine, reductions of mortality due to the

LD 50 of amytal were produced at three dose levels (200, 100 and 50 per cent of the LD 50 of strychnine), but they were not statistically significant, nor was a significant increase in mortality shown with the smallest dose of strychnine. The curve for the dimethyl compound shows that it behaves very like strychnine, but is, if anything, more active, significantly lower mortalities were produced by 50 and 100 per cent LD 50 doses. The diethyl compound, on the other hand, increased the mortality due to amytal, significantly at the highest dose (200 per cent. of its LD 50)

### III Local Anaesthetic Activity

Since it was thought possible that the amino substituted thiazole, diethyl-aminoethyl 2-amino-4-methyl-thiazole-carboxylate might possess local anaesthetic properties similar to those of procaine, it was tested for local anaesthetic activity by the method of Chance and Lobstein (1944). It was inactive at a concentration of 1 in 100, as was also diethylaminoethyl 4-methyl-thiazole-carboxylate.

### DISCUSSION

The piperidinoethyl, dimethylaminoethyl, diethylaminoethyl and  $\beta$ -diethylaminopropyl esters of 4-methyl-thiazole-5-carboxylic acid had approximately the same activity in inhibiting the contractions produced by acetylcholine in the isolated ileum (neurotropic action) but were only about 1/1,000 as active as Trasentin, the morpholinoethyl ester of 4-methyl-thiazole-5-carboxylic acid and the diethylaminoethyl ester of 2-methyl-thiazole-4-acetic acid were even less active. The compounds did not produce mydriasis. Their effect on spasm induced by barium chloride or histamine (musculotropic action) was of the same order as that of Trasentin. In this respect the piperidinoethyl, dimethylaminoethyl and diethylaminoethyl esters were more active than the morpholinoethyl or the  $\beta$ - and  $\gamma$ -diethylaminopropyl esters of 4-methyl-thiazole-5-carboxylic acid, the two isomeric propyl esters showed about the same activity. The diethylaminoethyl esters of 2-methyl-thiazole-4-acetic acid and thiazole-4-acetic acid had only a fraction of the activity of the corresponding ester of 4-methyl-thiazole-5-carboxylic acid, demonstrating the importance of direct attachment of the carboxylic group to the nucleus. The activity of the diethylaminoethyl ester of 2-amino-4-methyl-thiazole-5-carboxylic acid was 1/30 that of the corresponding ester of 4-methyl-thiazole-5-carboxylic acid, so that the introduction of an amino group materially reduced the musculotropic activity.

The diethylaminoethyl ester of 4-methyl-thiazole-5-carboxylic acid, although resembling Trasentin in its action on the isolated ileum, had a different effect on the isolated uterus of the rabbit or guinea-pig and, instead of relaxing the muscle, increased the tone in a manner similar to posterior pituitary extract or atropine. The substance was less potent, however, 2 mg. producing the same response as 0.05 I.U. posterior pituitary extract or 60  $\mu$ g. atropine sulphate. This ester had no effect on the ileal contractions induced by barium chloride or histamine hydrochloride *in vivo*.

Some of the esters were found to be stimulants of the central nervous system, producing convulsions in mice and guinea-pigs. Some of them reduced the duration of narcosis induced by amytal and paraldehyde, though the effect was markedly different with the different compounds.

Trasentin and Trasentin-6H are convulsants and respiratory stimulants (Graham and Lazarus, 1940), whilst atropine also stimulates the medulla and higher cerebral centres, though at the usual clinical dosage its effects are manifest only as moderate respiratory stimulation and slight vagal excitation. The thiazole compounds are less potent stimulants than leptazol or Trasentin. They act on the medulla, and the effect resembles that of leptazol rather than that of strychnine. The diethylaminoethyl ester of 4-methyl-thiazole-5-carboxylic acid was slightly effective in antagonizing the effect of a lethal dose of amytal, but the dimethylaminoethyl ester was not. Neither compound was as effective as leptazol in inducing central nervous stimulation, the lethal dose being close to the convulsive dose.

#### SUMMARY

1 The basic esters of 4-methyl-thiazole-5-carboxylic acid and of thiazole-5-acetic acid possess spasmolytic and analeptic properties.

2 The spasmolytic activity of these compounds is exhibited only *in vitro*, and is most marked against histamine spasm. A comparison of the different substances revealed that the dimethylaminoethyl ester of 4-methyl-thiazole-5-carboxylic acid was the most active, having an activity against histamine spasm approximately one-quarter that of Trasentin-6H, the diethylamino- and piperidinoethyl esters were only a little less active than the dimethylaminoethyl ester.

3 The diethyl- and dimethylaminoethyl esters and the  $\gamma$ -diethylaminopropyl ester of 4-methyl-thiazole-5-carboxylic acid produced in guinea-pigs and rabbits convulsions similar to those caused by leptazol and compounds acting on the mid-brain, although there was evidence of spinal involvement. These esters have analeptic activity, demonstrated by their ability to reduce the duration of anaesthesia in mice. The diethyl- and dimethylaminoethyl esters of 4-methyl-thiazole-5-carboxylic acid are, however, inferior to picrotoxin in this respect, not only is the reduction of paraldehyde anaesthesia less marked than the reduction of amytal anaesthesia, but antidotal activity is exhibited by the dimethyl compound over a smaller dose range than that found for picrotoxin.

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# THE TESTING OF DRUGS AGAINST EXOERYTHROCYTIC FORMS OF *P GALLINACEUM* IN TISSUE CULTURE

BY

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In 1938 James and Tate described a tissue phase of *P gallinaceum* in chicks which they called the exoerythrocytic phase of the parasite, and in 1945 Hawking succeeded in growing these forms in tissue culture. At the time of writing, similar forms have not been demonstrated in mammalian malaria, but it is likely that they do exist, and that the relapses common in benign malaria are due to their presence.

The tissue culture technique has been used as an *in vitro* method for testing the effect of drugs against the tissue phase of *P gallinaceum*. It is hoped that this test can be adapted so that the effect of drugs against the tissue phase of all types of Plasmodia can be investigated. A drug which was active against the tissue phase of the malaria parasite in this test would probably be a causal prophylactic and have a beneficial effect on the relapse rate of benign tertian malaria.

## METHOD

Ten-day-old chicks were injected with sporozoites of *P gallinaceum* and killed 7-8 days later, when they were heavily infected with tissue forms of the parasite. In a typical experiment the spleen from such an infected chick was removed aseptically minced finely in Tyrode's solution, and set up in Carrel flasks, each of which contained 3-4 coverslips using the method described by Hawking (1945). The fluid phase consisted of Tyrode's solution containing 20 per cent (v/v) chick serum and 3-4 per cent (v/v) chick embryo extract, 0.05 per cent phenol red was added to indicate changes in the pH of the medium. Penicillin was also added to make a final concentration of 3 units per ml as an additional safeguard against possible contamination by bacteria. (Later 0.5 unit/ml was used.) Drugs to be tested were dissolved in Ringer's solution sterilized by boiling, and appropriate concentrations made by serial dilution. 2.5 ml volumes of the fluid phase were then run into the flasks containing the infected spleen explants, followed by 0.5 ml volumes of the drug solutions—the control flasks receiving 0.5 ml volumes of sterile Ringer solution in place of drug. In this way a series of flasks was set up in which the same infected material was in contact with varying concentrations of several different drugs.

Under these conditions the final concentration of the drug in the flasks is less than the nominal concentration owing to the absorptive powers of the plasma and spleen explants used in the course of the experiments, the ratio of the nominal concentration to the final concentration may also vary because the amounts of spleen and plasma used were not

constant. This factor probably accounts for the disparity between some of the results obtained in different experiments using the same nominal concentrations of a particular drug. Frequent changing of the medium would probably obviate this effect, but it would be consuming of time and materials. In our experiments the medium was changed every 5 or 6 days when the pH of the medium became too low for cell growth to proceed favourably.

In the drug-free control flasks many parasites in all stages of schizogony were present after 7-10 days incubation at 37° C. When this was observed all the flasks in a series were sampled by withdrawing one or more of the coverslips from each aseptically, fixing them in methyl alcohol, staining with Giemsa, mounting, and examining them microscopically. In this way the effect of the drugs under test on the growth of the parasites in tissue culture could be observed. In cases where no parasites could be demonstrated microscopically, the result was confirmed by inoculation of aliquots of the fluid phase (0.5 ml - 10 ml.) taken from such flasks into chicks and observation of the presence or absence of subsequent infection.

The antimalarial action of the following compounds was investigated.

- 1 Sulphathiazole
- 2 Sulphadiazine
- 3 2-(*m*-aminobenzenesulphonamido)-pyrimidine (N'-2-pyrimidylmetanilamide in U S A)



- 4 Sontoquin (3-methyl-4-diethylaminoisoamylamino-7-chloroquinolme sulphate)
- 5 Streptomycin
- 6 Streptothricin

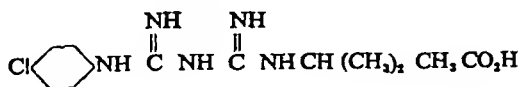
Compounds 3-6 were kindly supplied by the secretary of the Board for the Co-ordination of Malarial Studies, Washington.

The potency of streptomycin was stated to be 200 units per mg., and that of streptothricin to be 400 units per mg. In tests carried out in this laboratory using *B. coli* (strain No 88, Dr Felix), it was found that 0.05 mg. of streptomycin and 0.025 mg. of streptothricin just prevented the growth of the organism in 1 ml of Hartley's broth.

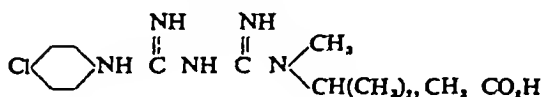
- 7 Quinine bisulphate
- 8 Mepacrine hydrochloride
- 9 Pamaquin dihydrochloride

This salt was kindly made for us by Dr T S Work from the hydroxynaphthoate of pamaquin.

- 10 Paludrine acetate (N<sub>1</sub>-*p*-chlorophenyl-N<sub>3</sub>-isopropylbiguanide acetate)



11 M4430 (*N*<sub>1</sub>-*p*-chlorophenyl-*N*<sub>2</sub>-methylisopropylbiguanide acetate)

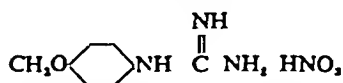


Compounds 10 and 11 were kindly supplied by Dr C. M. Scott, of Imperial Chemical (Pharmaceuticals), Ltd

12 Stilbamidine (Diamidinostilbene nitrate)

Kindly supplied by Dr Wien of May & Baker, Ltd

13 *p*-Anisylguanidine nitrate (V313)



This compound was made by Dr H King, F.R.S. It is the most active member of a series of guanidines and biguanides made for testing on malarial infections (King and Tonkin, 1946)

The concentrations quoted in the tables refer to the compounds or the salts of the compounds as listed above

## RESULTS

Results obtained in tests on these compounds using the tissue culture technique are given in Table I. Many of these compounds were toxic to macrophages, and a specific inhibitory effect of a drug on the exoerythrocytic forms of *P. gallinaceum* could only be recorded when the cells were observed to grow satisfactorily in the drug concentration used, while the parasites failed to grow.

Compounds of the sulphonamide type (sulphathiazole, sulphadiazine, and *m*-aminobenzenesulphonamidopyrimidine) had a considerable range of activity, i.e., the cells grew in high concentrations of the drugs, whereas the parasites were only able to grow in very low concentrations. *m*-Aminobenzenesulphonamidopyrimidine was slightly more toxic to macrophages than sulphathiazole and sulphadiazine. *p*-Anisylguanidine nitrate, a compound not containing the sulphonamide group but similar in structure to paludrine and M4430, also exerted considerable antiparasitic activity and was not toxic to the cells in the culture.

Of the other compounds tested, some activity was shown by streptomycin and streptothricin. The former was non-toxic to the cells in tissue culture in high concentrations and exerted an anti-parasitic effect when the concentration was above 100 mg/100 ml (=200 units per ml). Streptothricin was more toxic to the cells than streptomycin, but inhibited parasitic growth over a small range of concentrations not toxic to cells. The only other compound in the series to show any anti-parasitic activity was quinine. In all experiments except the second one (see Table I) the cell growth in contact with the higher concentrations

TABLE I

THE EFFECT OF DRUGS ON THE EXOERYTHROCYTIC FORMS OF *P. Gallinaceum* IN TISSUE CULTURE

++ Heavy growth of parasites + Good growth of parasites.

± No parasites seen microscopically but chicks infected

- No parasite growth T toxic, and SI T slightly toxic, to cells CP Poor growth of cells.

Drug	No of Expt	Concentration in mg /100 ml										Remarks	
Sulpha-thiazole		30.0	20.0	15.0	5.0	0.5	0.1	0.05	0.02	0.01	0.005	0.0	Approx. minimal effective concentration 0.05 mg./100 ml
	1	SI T		SI T								++	
	2				-	-	±					++	
	3		SI T					-				++	
	4							-				+	
	5							-	-	+	+	++	
Sulpha-diazine		100	50	25	10	0.5	0.2	0.1	0.05	0.0			Approx. minimal effective concentration 0.1 mg./100 ml
	1	-	-	-	-	-					+		
	2							-	-	-	+		
<i>m</i> -Amino-benzene-sulphon-amido pyrimidine		5.0	1.0	0.5	0.1	0.05	0.025	0.01	0.005	0.0			Approx. minimal effective concentration 0.1 mg./100 ml
	1	CP-	-		-					+	+	+	
	2				-					+	+	++	
	3		-	-	-	+	+	+	+	+	+	++	
	4				-	+	+	+	+	+	+	++	
Quinine bisulphate		25.0	12.5	5.0	2.5	2.0	1.0	0.5	0.0				Approx. minimal effective concentration 2 mg./100 ml Toxic to cells
	1	T	T								++		
	2				-						++		
	3						+		++	+	++		
	4					CP-				++			
	5					CP-				++			
	6					CP-	CP-			++			
	7			T			CP-			++			
	8			T	CP-					++			
	9						CP-		+	++			
Mepacrine		5.0	1.0	0.5	0.2	0.1	0.0					Inactive	
	1	T	T		±			+		++			
	2				-					++			
	3		T		T			++		++			
Pamaquin		0.5	0.1	0.05	0.02	0.01	0.0					? Slight action Approx. minimal effective concentration 0.05 mg./100 ml Very toxic to cells.	
	1	T	T							+			
	2		+		+		+			+			
	3		T							+			
Paludrine		2.0	1.0	0.5	0.2	0.1	0.05	0.02	0.01	0.005	0.0	No definite action	
	1		T	CP-		CP-	CP-				+		
	2						+		+	+	+		
	3				+	+	+	+			+		
	4	-	CP±	±	+	+	+	+			++		
	5	CP-	-	+							++		

TABLE I *continued*

Drug	No of Expt	Concentration in mg./100 ml								Remarks				
M4430		5.0	1.0	0.5	0.2	0.1	0.0	Inactive Toxic to cells						
	1	T	CP-			+	++							
	2		T	CP+	++	++	++							
	3			CP+	++	++	++							
	4			T	++	++	++							
<i>p</i> -Anisyl guanidine nitrate		5.0	2.0	1.0	0.5	0.2	0.1	0.05	0.0	Approx. minimal effective concentration 0.5 mg./100 ml				
	1	CP-		-	-		-		+					
	2			-	-	+		+	+					
	3	-	-	-					+					
	4	-	-	-		++	+		++					
Sontoquin		0.5	0.25	0.1	0.05	0.025	0.01	0.0	Inactive Very toxic to cells					
	1	T		T				++						
	2	T	T	T	T			++						
	3				+	+	+	++						
	4	T	CP++	++	++			++						
Stilbamidine		1.0	0.5	0.2	0.1	0.05	0.025	0.01	0.0	Inactive				
	1	-						+	+					
	2	CP-							++					
	3	CP-	CP+		+				+					
Streptomycin		500	250	100	50	25	5.0	2.5	2.0	0.4	0.1	0.01	0.0	Approx. minimal effective concentration 250 mg./100 ml (= 500 units per ml.)
	1								+	++	++		+	
	2												+	
	3				+	+	+	+					+	
	4	-	-		+								++	
	5		-	+	+								+	
Streptothricin		25.0	12.5	10.0	5.0	2.5	1.25	0.5	0.0	Approx. minimal effective concentration 2.5 mg./100 ml (= 10 units per ml.)				
	1	-	-	-		-	+	+	+					
	2	T		-					++					
	3					+	++	++	++					
	4				-	-	+	+	+					

\*A few parasites were seen in early samples taken from flasks but none in later ones, they appeared to have died off during the course of the experiment.

of the drug was poor, owing to the toxicity of quinine for cells of the macrophage type. It was therefore not possible to demonstrate a clear-cut anti-parasitic activity with this compound, but probably it exerts a slight effect on the growth of exoerythrocytic forms of *P. gallinaceum*.

No activity was exerted by mepacrine, ontoquin, M 4430, or stilbamidine. Pamaquin and paludrine both of which were expected to show activity, were also relatively ineffective. Both compounds were toxic to the cells, so that it is possible that if higher concentrations could have been tested, some anti-parasitic activity might have been detected.



TABLE II

THE INHIBITION OF THE ANTIPARASITIC ACTION OF SULPHONAMIDES

++ Heavy, + good growth of parasites. — No growth of parasites ?— A few disintegrating parasites seen but chicks not infected

Drug	Conc mg/100ml	Inhibitor	Conc mg./100ml	Number of Experiment					Ratio of conc. drug/ inhibitor
				1	2	3	4	5	
Sulpha- thiazole	5.0	PAB	0.5	++	++	++			500
			0.1		++			+	
			0.05		++			+	
			0.02					+	
			0.01					+	
			0.005					—	
			0.0	—	—		—	—	
	0.0	PAB	0.0	++	++	+	++	++	0.1
			10.0			+			
	5.0	MAB	5.0	—					
			0.0	—					
	0.0	MAB	0.0	++	+				
			10.0		+				
<i>m</i> -Aminoben- zene-sulphon- amido-pyri- midine	1.0	PAB	1.0	—					
			0.0	—					
	0.0	PAB	0.0	++					
	0.5	PAB	10.0		+				
			5.0		+		—		
			1.0				—		
			0.5			—			
			0.1			—			
			0.0		—	?—	—		
	0.0	PAB	0.0		+	++	++		
			10.0		+				

\* A few parasites were seen in early samples taken from the flasks, but none in later ones. they appeared to have died off during the course of the experiment

## INHIBITION OF ANTI-PARASITIC ACTIVITY

Experiments were also set up to demonstrate the inhibition of the anti-parasitic activity of compounds of the sulphonamide type. The drugs used were sulphathiazole and *m*-aminobenzenesulphonamidopyrimidine and the corresponding inhibitors were *p*-aminobenzoic acid (PAB) and *m*-aminobenzoic acid (MAB) (Table II). 0.01 mg/100 ml PAB inhibited the activity of 5 mg/100 ml sulphathiazole, one molecule of the inhibitor therefore being equivalent to approximately 270 molecules of the drug.

No inhibition of the activity of the *m*-sulphonamide by MAB could be demonstrated, but high concentrations of PAB had a slight action in this respect.

## THE EFFECT OF DRUGS ON CULTURES ALREADY GROWN

The effect of sulphathiazole on parasites already grown in tissue culture was also investigated. For these experiments cultures of spleen containing exoerythrocytic forms were taken after incubation for 7-14 days at 37° C in drug-free medium, at which time they showed vigorous growth of cells and parasites.

TABLE III

THE EFFECT OF SULPHATHIAZOLE ON EXOERYTHROCYTIC FORMS OF *P. Gallinaceum* ALREADY GROWING IN TISSUE CULTURE

Expt.	Age of culture when drug added Days	Conc of sul- phathiazole mg./100 ml	Conc. of PAB mg /100 ml	Days of exposure to the drug						
				1	3	4	5	6	7	
a	9	10.0		+	—		—			
	8	5.0		+	+	+	?—		—	
		2.0			+		?—		?—	
		1.0		+	+	+	?—		?—	
		0.5		+	+	+	?—		—	
		0.0		++	++	++	++		+	
b	11	5.0			+	+		—	—	
		1.0			+	+		—	—	
		5.0	20.0		+	+		+	±	
			10.0		+			+	±	
			5.0		+	+		+	+	
		0.0	0.0			++	++		+	+

++ Heavy parasite growth + Some parasite growth.

± No parasites seen microscopically but chicks infected.

?— A few disintegrating parasites seen Chicks not infected

The fluid phase was removed and replaced with fresh medium containing varying concentrations of drug. Slips were removed, fixed, stained, and examined after 1, 3, 4, 5, 6, and 7 days' incubation at 37° C in contact with the drug.

The protocols of a typical experiment are given in Table IIIa. It will be seen that the parasites were all dead after 5 days' exposure to all concentrations of sulphathiazole tried.

Table IV shows percentage counts of parasites in different stages of development from cultures in contact with 0-10 mg/100 ml of sulphathiazole for 1-5 days. The increasing scarcity of parasites in the cultures with increasing time of exposure is shown by the drop in the total number of parasites counted.

TABLE IV

THE EFFECT OF SULPHATHIAZOLE ON DIFFERENT STAGES OF PARASITES GROWING IN TISSUE CULTURE

Sulpha- thiazole mg./100 ml	Time of exposure to drug Days	Percentage numbers of different forms of parasite				Total number of parasites counted
		Schizonts 8 nuclei	Schizonts 3-8 nuclei	Schizonts 1-2 nuclei	Groups of Merozoites	
10.0	1	5.4	15	79	0	186
	2	1.58	19	80	0	63
	4	100	0	0	0	16
5.0	1	31.5	11.6	57	0	156
	3	20.5	51	23.5	0	131
	4	61	39	0	0	46
2.0	3	0.54	41.5	58	0	186
	5	36.5	63.2	0	0	30
1.0	1	39	18.6	41	1.5	194
	3	3.5	88	2.9	0	173
	4	100	0	0	0	31
0.0	-2*	33	45	24	0	95
	0†	8.45	3.9	86.5	1.3	154
	3	35.5	29.5	31.7	3.7	164
	5	11.1	59.5	27.7	1.58	126

\*Two days before addition of sulphathiazole.

†Immediately before addition of sulphathiazole.

in cultures after 5 days' exposure as compared with the total after one day's exposure and with that in the control flasks. Approximately the same length of time was spent in examining the sample from each flask. It will also be seen that the number of small forms became progressively smaller on successive days of exposure to the drug until on the fourth day they had disappeared altogether, while abundant small forms and merozoites were still found in the control flasks. Sulphathiazole apparently has a gradual effect on the parasites, reducing their power of division.

The larger schizonts still remaining after 3 days' exposure showed definite pathological changes. The chromatin coalesced into lumps, the parasite envelope became indistinct and the cytoplasm became vacuolated and lost its power to take up stain. In later stages the chromatin appeared as indistinct collections of small granules (Fig. 1). These changes were associated with a loss of the ability of the parasites to infect chicks.

The effect of PAB on drug activity was also investigated in this connection, the inhibitor being added at the same time as the drug. Inhibition of drug

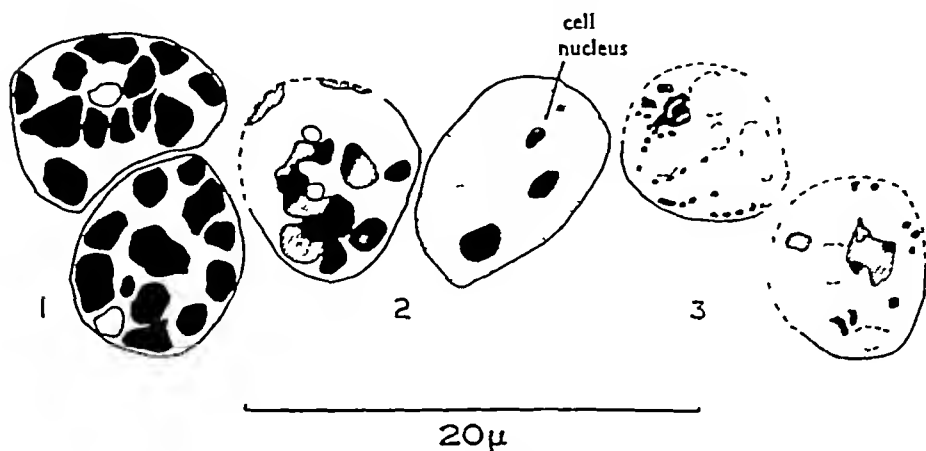


FIG. 1—Drawn by I M T (magnification as shown by scale)

- 1 Parasites before treatment with drug.
- 2 Parasites after exposure for 1 day to sulphathiazole (10 mg./100 ml) Chromatin coalescing into lumps
- 3 Parasites after exposure for 5 days to sulphathiazole (10 mg./100 ml) Chromatin in small granules Envelope vague, vacuolated cytoplasm

activity was not so marked as in the experiments given in Table II, the parasites being reduced in numbers compared with those in the control flasks, and also showing pathological changes. However, they maintained their ability to infect chicks for a longer period than those parasites in contact with sulphathiazole alone, showing that some inhibition of drug activity had taken place.

The protocols of an experiment of this type are given in Table IIIb.

#### DISCUSSION

A method for the *in vitro* testing of drugs against the tissue phase of *P. gallinaceum* is described and the results obtained on several compounds tested by this method are given. The technique is probably too elaborate to be used in a routine screening test, but it might be employed with advantage in the further investigation of promising compounds singled out by the *in vivo* screening tests.

at present in force. The method is limited by the fact that many compounds are very toxic to macrophages in tissue culture, and in these cases it is often difficult to detect any direct effect of the drug on parasite growth as distinct from the effect on the growth of the parasites due to the poor survival of the cells which form their substrate. The solubility of the compounds to be tested is another limiting factor.

The antiparasitic activity of sulphathiazole is inhibited by PAB. The equivalence of one molecule of the inhibitor to 270 molecules of the drug cannot be regarded as a true quantitative relationship, because the medium in which the reaction took place contained many natural sulphonamide inhibitors, such as serum, plasma, tissue extracts, etc.

Previous work on this relationship carried out with bacteria shows considerable variation in the sulphathiazole/PAB ratio. Rose and Fox (1942), using *B. coli* growing in a synthetic medium, found one molecule of PAB to be equivalent to 4 molecules of sulphathiazole, while Landy and Wyeno (1941), using a staphylococcus growing in a heart infusion broth, found that one molecule of the inhibitor neutralized 2,000 molecules of the drug. Our figure of 1-270 falls between these two extremes.

Inhibition of the antiparasitic activity of *m*-aminobenzenesulphonamidopyrimidine by MAB in a prophylactic test in chicks had been reported by Dr S Brackett (private communication). It was therefore expected that this reaction could be demonstrated in tissue culture. No inhibition was in fact obtained with this combination of reagents, but PAB in a high concentration had a slight effect on the activity of the *meta*-sulphonamide. We have also been unable to confirm this finding of Brackett's in experiments on chicks.

Our experiments in which sulphathiazole was placed in contact with exoerythrocytic forms already grown in tissue culture showed that the drug probably reduced the power of division of the parasites. A similar effect was noted in the case of erythrocytic forms of *P. gallinaceum* in chicks treated with sulphadiazine by Brackett, Waletzky, and Baker (1945).

#### SUMMARY

(1) The effect of drugs on the exoerythrocytic forms of *P. gallinaceum* has been investigated using an *in vitro* technique. Explants of chick spleen infected with tissue forms of the parasite were grown in Carrel flasks in contact with a solution of the drug in a nutrient medium, and the effect of the drug on parasite and cell growth was observed microscopically.

(2) The following compounds inhibited the growth of the parasite and were comparatively non-toxic to the macrophages: sulphathiazole, sulphadiazine, *m*-aminobenzenesulphonamidopyrimidine, streptothricin, streptomycin, and *p*-anisylguanidine nitrate. Quinine showed a slight antiparasitic activity but was toxic to macrophages. Mepacrine, stilbamidine, M 4430 ( $N_1$ -*p*-chlorophenyl- $N_2$ -methylisopropylbiguanide), ontoquin, pamaquin, and paludrine were very toxic.

to the cells and were inactive in the highest concentrations tolerated by the macrophages

(3) The antiparasitic activity of sulphathiazole could be inhibited by *p*-aminobenzoic acid, one molecule of the inhibitor corresponding to 270 molecules of the drug. No inhibition of the *m*-sulphonamide, *m*-aminobenzene-sulphonamidopyrimidine, by *m*-aminobenzoic acid could be demonstrated.

(4) When parasites, already grown in tissue culture, were exposed to sulphathiazole, the drug appeared to reduce the power of division of the parasites. After 4 days' exposure to the drug no small forms were found and the larger schizonts became progressively more degenerate. After more than 4 days' exposure the parasites lost the ability to infect chicks.

(5) The tissue culture technique may not be suitable for large-scale routine drug screening tests, but would be useful for the further investigation of promising compounds.

Grateful acknowledgements are due to Dr F. Hawking for help and advice, and to Miss R. J. Berson and to Miss P. Davey for technical assistance.

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# EXPERIMENTAL DIABETES THE EFFECT OF LIGATION OF THE PANCREATIC DUCT UPON THE ACTION OF ALLOXAN IN RABBITS\*

BY

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It is known from the work of Shaw Dunn and his collaborators [(1943 (a) and (b), 1944) and Duffy (1945)], Bailey and Bailey (1943) and others that it is possible, by the intravenous administration of alloxan, to produce in rabbits a selective necrosis of the islets of Langerhans and a condition indistinct from diabetes mellitus. We have studied the effect of alloxan administration in rabbits at various times after ligation of the main pancreatic duct. When sufficient time is allowed to elapse after the operation to permit the acinar tissue to degenerate, alloxan, subsequently administered, produces at most slight changes in the surviving islet cells and no diabetes. This result cannot yet be conclusively explained, but strongly suggests, among other possibilities, that the acinar tissue of the pancreas is in some way involved in the action of alloxan upon the islets.

## EXPERIMENTAL

The rabbits used in our experiments were adult males and females of various breeds. They were bedded on hay in wire-mesh cages and maintained on a standard composite diet (Thomson, 1936) supplemented with cabbage. They were starved for 24 hours before operation or alloxan administration. Blood sugar was determined by the method of Hagedorn and Jensen on 0.2 ml. samples of blood withdrawn from an ear vein. Alloxan was in all cases given in 4 per cent solution in physiological saline, by slow intravenous injection into a marginal ear vein.

The whole pancreas was taken for histological examination, fixed in Zenker-formol and embedded in paraffin. Sections were stained with (a) haematoxylin-eosin, (b) Wilder's

\* A preliminary communication on this subject was given by one of us (A.L.W.) at the meeting of the British Pharmacological Society, July, 1945.

silver impregnation method (c) a modification of Mallory's aniline blue method for the differentiation of  $\alpha$  and  $\beta$  cells in the islets of Langerhans, and occasionally with chlorazol black for elastic fibres

For the characterization of the changes in the pancreas produced by the various procedures to which rabbits were submitted, certain features of the normal rabbit pancreas are pertinent. In haematoxylin-eosin (H E) preparations the islets are extremely numerous and stand out in the lobules as pale staining round or oval bodies against the darker staining acinar tissue (Plate I, Fig 1). Under higher magnifications they are seen to be composed of anastomosing cords of cells, the outlines of the individual cells being rather obscure (Plate I, Fig 2). The cytoplasm is pale, but even in the H E preparations it is often possible to distinguish between certain cells at the periphery stained faintly eosinophilic and more numerous centrally placed cells in which the cytoplasm is stained a sombre greyish blue. The nuclei of both  $\alpha$  and  $\beta$  cells are round or oval, show an even powdered distribution of chromatin particles, and contain a minute nucleolus. A few fibroblasts may be present in the islet tissue and occasionally red blood corpuscles are seen in minute sinusoidal channels. With silver impregnation methods the pancreas is shown to have a scanty and loose inter-lobular stroma while the acini are mapped out by virtue of an enclosing intra-lobular argyrophil reticulum (Plate I, Fig 3). The latter also forms a compact basket-work around the islets but not in the sense of a capsule, only very few fibrils penetrate into the centre. With Mallory's method the  $\alpha$  cells, present mainly at the periphery of islets, have orange to red granular cytoplasm, while the  $\beta$  cells contain granules stained a much fainter colour and predominate in the centre. Mitoses are rarely observed in the islet cells.

#### *The effects of alloxan in the unoperated rabbit*

In the unoperated rabbit an adequate dose of alloxan produces a characteristic sequence of changes in the blood sugar concentration. These effects were described in detail by Shaw Dunn (*loci cit*) and others, in other animals as well as rabbits, and our observations in this direction add nothing new.

A typical result is shown in Fig 1 which represents the blood sugar concentration of rabbit W 5 (1.9 kg) before and after a single intravenous dose of 200 mg/kg of alloxan.

A sharp initial rise was followed within an hour or two by a fall, during which the animal passed into hypoglycaemic convulsions and coma. Death at this stage was averted by the repeated administration of glucose and there finally ensued a chronic state of hyperglycaemia resembling that of diabetes mellitus.

The pathological effects on the pancreas are briefly as follows. Within a few hours of the injection, minor degenerative changes appear in the islet cells—obscurity in the staining of the cytoplasm and nuclear hyperchromatism, dislocation of cells from those adjacent and the formation of small spaces—limited mainly, if not solely, to the  $\beta$  cells of some isolated islets (Plate I, Fig 4). Later



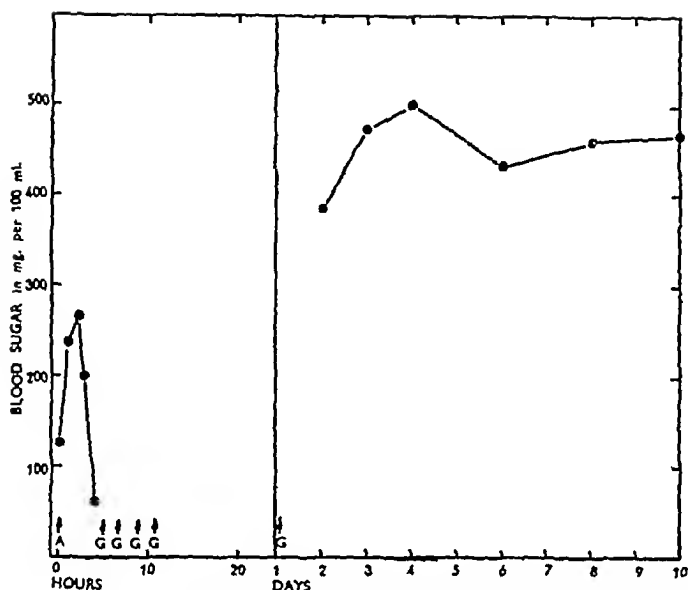


FIG 1—Blood sugar changes in unoperated rabbit W 5, given alloxan, 200 mg/kg., i.v., at A. Glucose, 2.5 g. in 50 per cent aqueous solution, given i.v., at G. Rabbit killed after 10 days.

definite coagulative necrosis usually develops (Plate I, Fig 5). Few islets escape damage and some of the  $\alpha$  cells may become involved, so that finally in certain cases it becomes difficult to find normal islet tissue in a number of sections of pancreas. The rate at which these changes occur and their final extent vary from animal to animal, even when the same dose of alloxan is given to all.

In our experiments a single dose of 100 mg per kg. alloxan was given to each of four rabbits, and a dose of 200 mg per kg. to a further ten. Thirteen responded with hyperglycaemia followed by a fall in blood sugar to levels at which convulsions supervened. The highest values recorded during the initial hyperglycaemic phase ranged from 155 to 464 mg/100 ml. Three of the animals were given glucose during the hypoglycaemic phase and subsequently developed diabetes with blood sugar levels varying between 350 and 600 mg/100 ml. These three animals were killed at 5, 6 and 10 days respectively after the administration of alloxan and the pancreas removed for histological examination. The pancreas was taken from six of the others at death. In each case definite degenerative changes were found in the islet cells. Four animals died overnight and autolysis was too marked to permit of detailed observations.

In this series only one animal—one which had received the higher dose of alloxan—failed to exhibit marked changes in the blood sugar concentration. This animal was killed seven days after dosing when it had a blood sugar of 115 mg/100 ml. No significant lesions were found in the pancreas. Other workers have encountered the occasional animal which fails to respond to alloxan.

*The effect of alloxan at various times after ligation of the pancreatic duct*

Young rabbits 1 to 1.5 kg in weight, were anaesthetized with ether and a two to three inch laparotomy incision made in the midline from a point about one inch caudal to the xiphoid process. The pancreatic duct was located and a single silk ligature tied around it as near as possible to its point of entry into the duodenum (In the rabbit the pancreas consists of lobules scattered in the mesentery, a large duct enters the intestine below the bile duct, while the smaller duct is so atrophied that it is almost impermeable) The abdominal muscles and skin were closed separately. Recovery was uneventful and the animals remained in good health and gained in weight. The changes in the pancreas in two rabbits killed at 30 and 56 days respectively after the operation are briefly described below

*Rabbit W.23, killed 30 days after ligation of the duct.*

The gland was markedly pale and very much reduced in size. Little more remained than a fibrous cord of tissue surrounding the dilated main duct proximal to the ligature. Histologically the lobules were atrophied and so reduced in size that in any one low power field the remnants of about six were visible (Plate I Fig 6). The normal inter-lobular tissue showed a gross increase in collagenous fibres in which persisting normal inter-lobular ducts were present. The acinar tissue proper had undergone extreme atrophy, the acini showed dilated lumina with a low cubical type of epithelium and occasionally contained hyaline material. Throughout the fibrosed gland the islets, both intra- and inter-lobular in position, stood out clearly as irregular, round or oval masses of cells in which both  $\alpha$  and  $\beta$  types could be distinguished (Plate II, Fig 7)\*. In silver preparations these islets were seen to be enclosed in a thick felt work of argyrophil reticulum (Plate II, Fig 8).

*Rabbit W.38, killed 56 days after ligation of the duct.*

More advanced changes were found in the pancreas of the same nature as those described above, sclerosis was much more severe, islet tissue was normal.

(a) *Short term ligation. Effect of alloxan.* Two rabbits, W.55 and W.59, were each given a single dose of 200 mg/kg alloxan on the day after ligation of the duct. The blood sugar changes were indistinguishable from those in unoperated animals treated with alloxan.

One rabbit, W 55, died in hypoglycaemic convulsions overnight, the other was kept alive during the hypoglycaemic phase by repeated injections of glucose and subsequently developed a persistent hyperglycaemia. When killed four days after the administration of alloxan, it had a blood sugar concentration of 552 mg./100 ml. Pathological examination of the pancreas showed the lobules of the pancreas to be greatly shrunken, so that in any one low power field as many as about eight could be seen (Plate II, Fig 9). The acinar tissue showed the initial

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\* These changes were exactly similar to those described in the early original work of Schultz (1900), Ssobolew (1902), and McCallum (1909).

changes of the same nature as that described for rabbit W 23, i.e., dilation of ductules due to retention stasis, but no gross fibrosis of the inter-lobular stroma. Islets were difficult to find, and many must have disappeared entirely. Those remaining (Plate II, Fig 10) were shrunken and showed changes such as have been described for unoperated animals treated with alloxan.

(b) *Long term ligation* A number of rabbits were treated with alloxan at longer times, ranging from 23 to 58 days, after ligation of the duct, the effects were very different from those in unoperated rabbits and are described below.

*Rabbit W 26* Alloxan, 100 mg /kg., given 30 days after duct ligation

No significant change in the blood sugar concentration occurred within the first seven hours of dosing or at twenty-four hours (Fig 2), and at this point, therefore, a further dose of 200 mg /kg was given. No significant change occurred subsequently, and when the animal was killed eight days later the blood sugar was still normal.

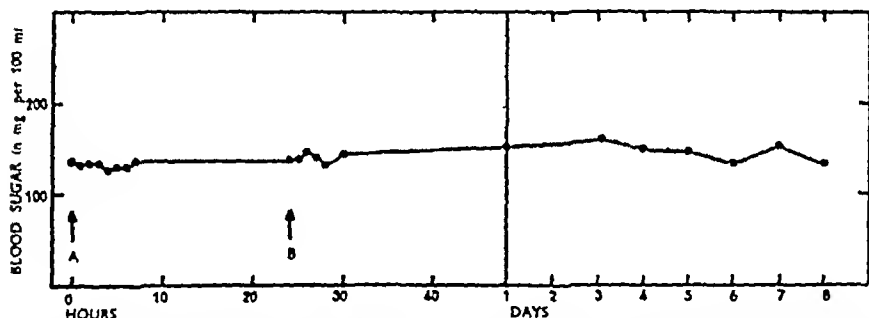


FIG 2—Blood sugar changes in rabbit W.26, pancreatic duct ligated 30 days before the administration of alloxan, 100 mg./kg., i.v., at A. Alloxan 200 mg./kg i.v given at B. Rabbit killed at 10 days.

The pancreas showed a severe sclerosis such as has been described for rabbits W 23 and W 38. The islets were easy to find and prominent amidst grossly thickened collagenous stroma or as solid cellular bodies in atrophic parenchyma (Plate II, Fig 11). Normal  $\alpha$  and  $\beta$  cells were present.

*Rabbits W 27, W.36 and W.37* Alloxan, 200 mg per kg, given 34, 53 and 51 days respectively after duct ligation. Killed four days later. For blood sugar changes see Fig 3.

In rabbit W 27 severe pancreatic sclerosis was found. The islets were prominent in the fibrosed stroma and along the grossly thickened wall of the main duct, and most of the atrophic lobules showed one or two. Both  $\alpha$  and  $\beta$  cells could be clearly distinguished and showed no evidence of degeneration.

In rabbit W.36 very advanced pancreatic sclerosis was found. Scattered

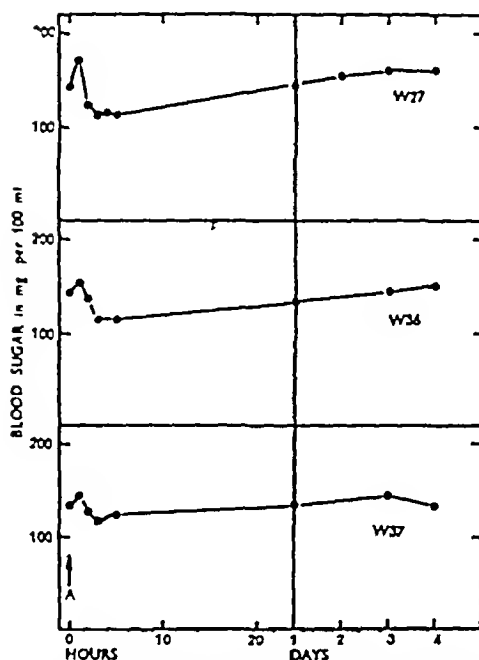


FIG 3—Blood sugar changes in rabbits W.27, W.36 and W.37, pancreatic duct ligated 34, 53 and 51 days, respectively, before the administration of alloxan 200 mg./kg. i.v., at A. Rabbits killed 4 days later

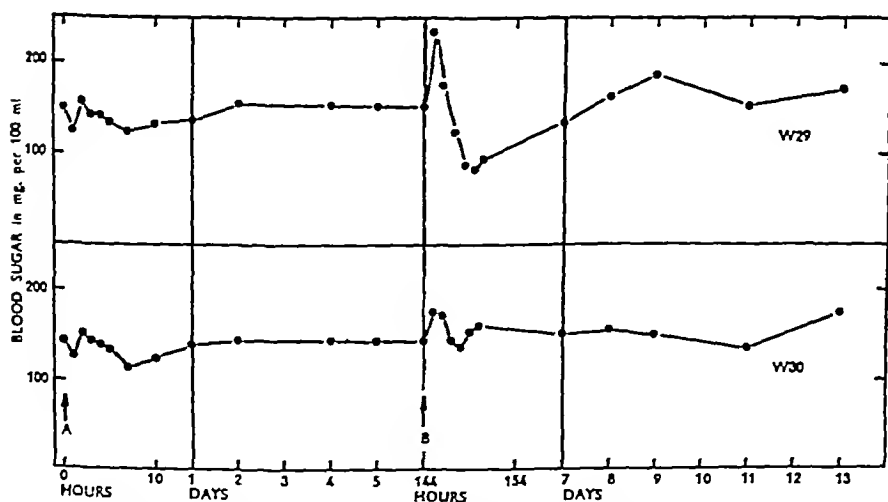


FIG 4—Blood sugar changes in rabbits W.29 and W.30 pancreatic duct ligated 31 and 23 days, respectively, before the administration of alloxan 200 mg./kg., i.v., at A. Alloxan 300 mg./kg., i.v., given at B, 6 days later. Rabbits killed 13 days after the first injection

throughout the abundant collagenous tissue normal islets stood out as small and large, round or irregular compact masses of cells (Plate II, Fig 12)

In rabbit *W 37* the appearance of the pancreas was almost identical with that in rabbit *W 36*

*Rabbits W 29 and W 30* Alloxan, 200 mg /kg, given 31 and 23 days respectively after duct ligation

Blood sugar changes up to six days after dosing were slight (Fig 4) and at this point the animals were each given a further dose of 300 mg per kg. The blood sugar changes thereafter were of the same general nature as those produced by alloxan in unoperated animals but were less pronounced and there was no marked persistent hyperglycaemia. The animals were killed seven days after the second injection

Advanced pancreatic sclerosis was found in both cases. Almost as much normal islet tissue was present as remnants of atrophied parenchyma. Peri-ductal islet tissue was particularly prominent along the course of the main duct.

*Rabbits W 40 and W 73* Alloxan, 400 mg per kg, given 58 and 39 days respectively after ligation of the duct. For blood sugar changes see Fig 5

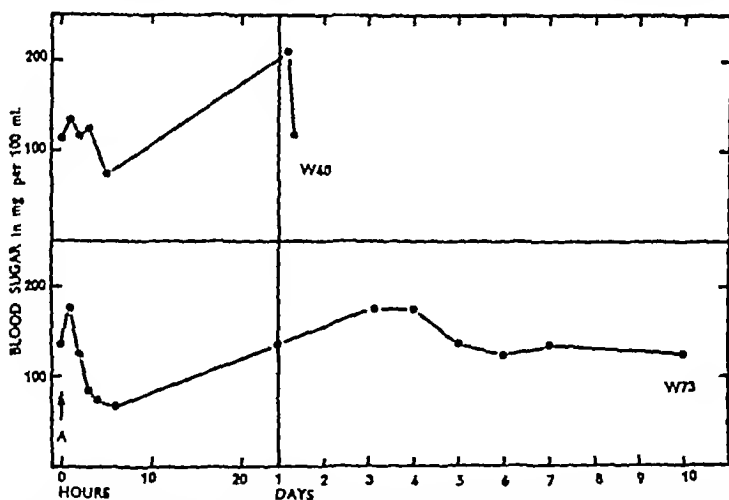


FIG 5—Blood sugar changes in rabbits *W 40* and *W 73*, pancreatic duct ligated 58 and 39 days, respectively, before the administration of alloxan 400 mg /kg, i.v. at A. Rabbit *W 40* died after 24 hours, rabbit *W 73* was killed 10 days after the injection

Rabbit *W 40* had a blood sugar concentration of 210 mg per cent on the morning following the day of injection. It died at 4 p.m. on the same day. The blood sugar concentration determined on blood taken from the heart immediately after death was 118 mg /100 ml. Post-mortem examination revealed hydrothorax, hydropericardium and pulmonary oedema. Histologically the lungs showed areas of alveolar haemorrhage and oedema, and the kidneys a diffuse

patchy condition of tubular necrosis, particularly affecting the proximal convoluted tubules, with haemoglobin casts in the lower parts of the nephron. Pancreatic sclerosis was more advanced than that seen in any other animal. The gland had shrunk to a very small fibrous mass in which were ducts with cystic dilatations. There was calcification of the stroma, of the intima and media of the blood vessels and of some parts of the walls of the persisting ducts. There was a moderate degree of lymphocytic infiltration. Normal islet tissue was abundant and prominent. The death of this animal cannot be attributed to damage of the pancreas (The renal lesion in this animal is similar to that described by Dunn *et al* (1943b) as a nephrotoxic effect of alloxan, and, as they stated then, is probably due to the concentration of the agent in the tubules in process of excretion. This, however, has no direct significance as far as the main object of this paper is concerned.)

Rabbit W 73 was killed ten days after being given alloxan. The pancreas was similar to that of rabbit W 40, but there was neither calcification nor lymphocytic infiltration. Islet tissue did not appear to be so prominent in this animal, and in a few islets minor degrees of cellular damage were observed.

#### DISCUSSION

The results described above leave no doubt that ligation of the main pancreatic duct in the rabbit leads in time to almost complete resistance to alloxan in so far as its action upon the islet cells, with the consequent production of diabetes, is concerned.

The operation leads to atrophy and replacement fibrosis of the pancreatic parenchyma proper while the islets remain but slightly affected, even after very long periods. With the development of sclerosis they become enclosed in a thick felt-work of argyrophil reticulum and appear perhaps somewhat more compact than in the normal animal, but most of their cells remain normal in appearance. These changes are exactly similar to those occurring in the so-called chronic interstitial pancreatitis in man, which commonly results from occlusion of the duct by calculi (see Warren, 1930). The fact that the fasting blood sugar remained within but slightly elevated limits after ligation of the duct is further evidence for the persistence of healthy functioning islet tissue. The mean fasting level of 16 normal animals of our stock was 118.4 mg/100 ml, with extremes of 105 and 130, while that of the eight animals in our experiments in which the duct had been ligated for times ranging from 23 to 58 days was 136.1 mg./100 ml, with extremes of 110 and 149.

In rabbits in which the duct had been ligated for 23 or more days, alloxan in doses up to 400 mg/kg produced in most cases comparatively slight fluctuations in the blood sugar level. In no case were hypoglycaemic convulsions or a final persistent hyperglycaemia observed. In no case, moreover, was marked damage to the islet cells encountered. (The possibility of alloxan having caused damage to some islets and of their subsequent regeneration cannot, of course be excluded.)



FIG 1—Pancreas normal rabbit Showing six pale staining islets standing out against the darker stained parenchyma

15 46Q — H E  $\times 75$

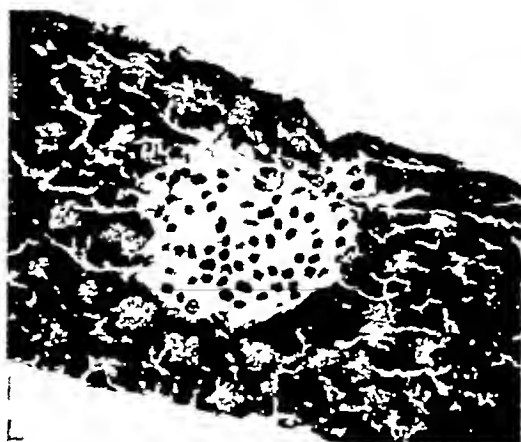


FIG 4—Pancreas rabbit Effects of alloxan Rabbit killed after 7 hours Shows minor degenerative changes and hyperchromatism of the  $\beta$  cells of the islet Rabbit W34

15 46E— H E  $\times 400$

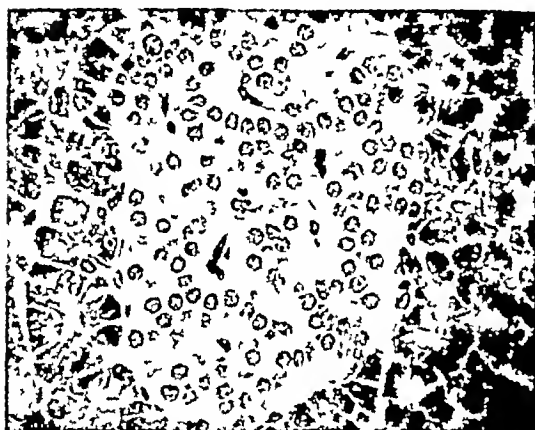


FIG 2—Pancreas normal rabbit Islets showing anastomosing cords of cells, cytoplasmic outlines obscured, round nuclei with even powdered distribution of chromatin Minute vascular channels Field from Fig 1

H E  $\times 400$

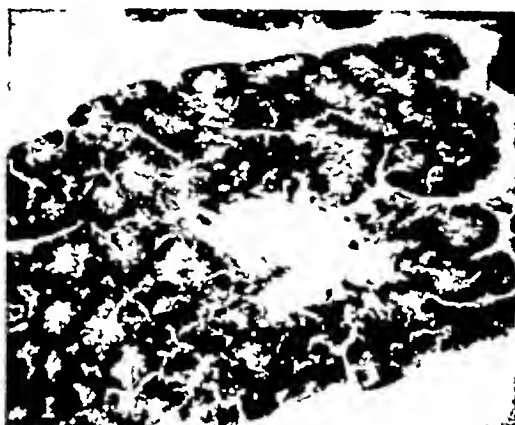


FIG 5—Pancreas rabbit Effect of alloxan Showing definite coagulative necrosis of islet Rabbit W35

15 46G — H E  $\times 400$



FIG 3—Pancreas normal rabbit Showing distribution of collagenous fibres and reticulum The islets contain no reticulum

Wilder's method and van Gieson  $\times 400$



FIG 6—Pancreas, rabbit Duct ligated animal killed after 30 days Showing atrophic lobules dilatation of persisting acini and replacement fibrosis Normal islet tissue Rabbit W23

15 46A — H E  $\times 75$

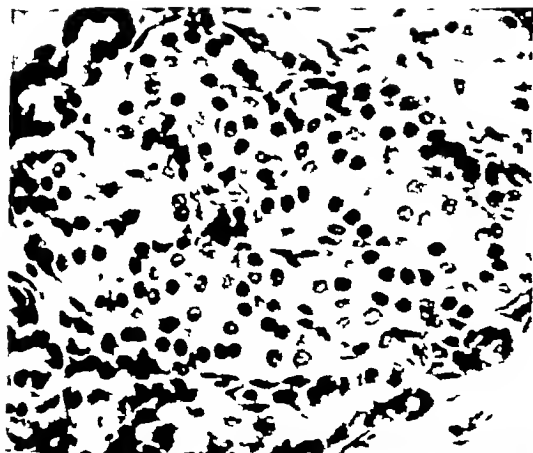


FIG 7—Pancreas, rabbit, from same animal as Fig 6  
Islet showing normal appearance

H.E.  $\times 400$

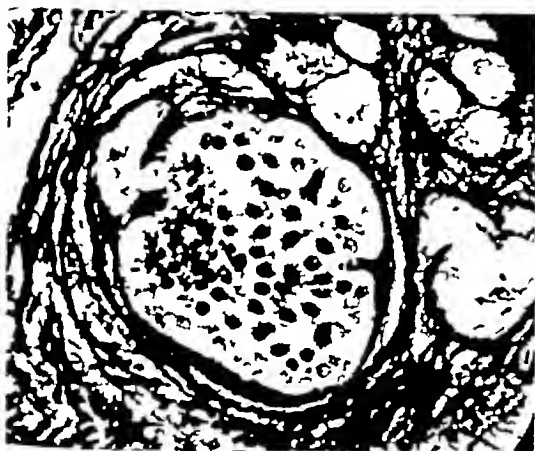


FIG 8—Pancreas, rabbit, from same animal as Figs 6  
and 7. Showing fibrosis and the thick felt work of fibrils  
around islet

Wilder's method and van Gieson  $\times 400$



FIG 9—Pancreas, rabbit. Effect of alloxan injected  
shortly after ligation (24 hours) of duct. Atrophic  
lobules, beginning of fibrotic changes. Rabbit W.59  
15 46H—H.E.  $\times 75$

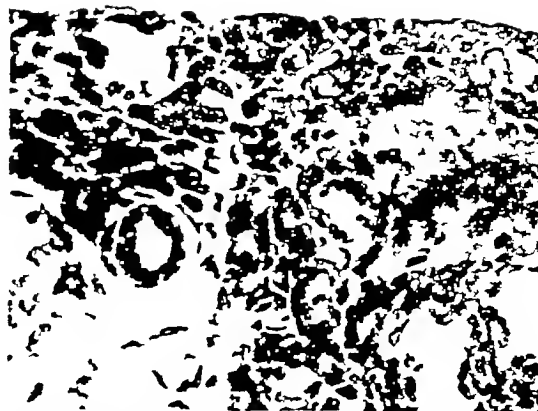


FIG 10—Pancreas, rabbit from same animal as Fig 9  
Showing islet under high magnification with necrotic  
changes

H.E.  $\times 400$



FIG 11—Pancreas, rabbit. Effect of alloxan after pro-  
longed period of ligation of duct (30 days). Showing  
condition of pancreatic sclerosis but normal islets.  
Rabbit W.26  
15 46—H.E.  $\times 400$

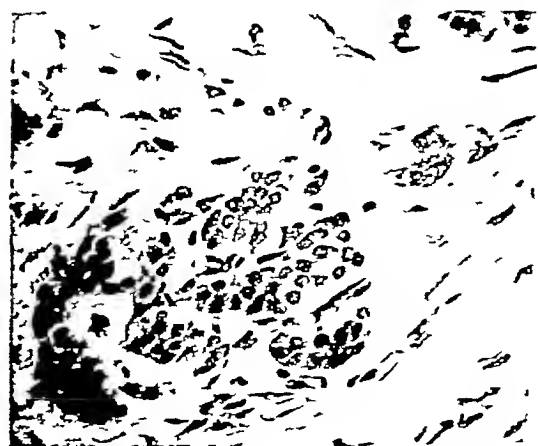


FIG 12—Pancreas, rabbit. Effect of alloxan after  
prolonged ligation of duct (53 days). Very advanced  
pancreatic sclerosis. Normal islets. Rabbit W.6  
15 46K—H.E.  $\times 400$



Since resistance to alloxan develops *pari passu* with atrophy and replacement fibrosis in the acinar tissue it seems reasonable to conclude that it is a direct result of these changes

The nature of the mechanism involved is not clear. It may be simply physical—explanations of this kind have been given traditionally, though without much real evidence to support them, for somewhat analogous phenomena. The fibrosis which develops in the duct-ligated gland may produce such a degree of ischaemia that alloxan subsequently administered cannot reach the islet cells in sufficient concentration to produce its necrotic effect before it is fixed or destroyed in other tissues. It is known that alloxan disappears very rapidly from the blood stream. Leech and Bailey (1945) were able to demonstrate the compound in the blood for only five minutes after intravenous injection and Gomori and Goldner (1945) showed that in the dog its toxic activity towards the islet cells was limited to the few minutes immediately after administration. They clamped off a portion of the pancreas during and for periods of from one to six minutes after intravenous injection of alloxan and found that this procedure protected the clamped off portion of the gland from damage, while in the unclamped parts necrosis developed in the  $\beta$ -cells of the islets. This physical explanation is perhaps further supported by our observation that massive doses of alloxan produced some damage in the surviving islet cells of one duct-ligated rabbit. The fact remains, however, that in all our experimental animals the vascular supply to the pancreas was sufficient to prevent general ischaemic necrosis of the sclerotic organ.

If we reject this explanation we can only conclude from our results that the acinar tissue is in some way involved in the action of alloxan upon the islets. The precise nature of any such involvement remains undetermined.

It may be (a) that alloxan facilitates the access of trypsinogen in an activated form to the islet cells and that the enzyme is immediately responsible for their necrosis, or (b) that it is not alloxan itself but some product of its interaction with the acinar secretion that causes damage to the islets.

The participation of trypsin appears to be excluded by observations of Rich and Duff (1936). They found that trypsin, trypsinogen, or crude pancreatic extracts injected into the main pancreatic duct of the dog produced at first mainly vascular damage—necrosis of vessel walls and haemorrhage—and ultimately a condition similar to acute haemorrhagic pancreatitis in man, with extensive general damage to the organ. They showed also that bile, injected similarly in quite small amounts, was sufficient to cause rupture of the terminal ductules and acini and liberate into the inter-acinar tissue some substance in sufficient quantity to cause similar lesions. There was no evidence that any of these procedures resulted in selective damage to islet tissue. If alloxan did in fact activate trypsinogen and in some way facilitate its access to the islet cells one would expect it to produce widespread pancreatic necrosis and haemorrhage—effects, that is, similar to those observed by Rich and Duff. The selective nature of the damage produced by alloxan would thus appear to exclude the possibility

of its acting in this way. It does not exclude the possibility that, as our findings strongly suggest, it is alloxan in combination with actively functioning acinar tissue which is responsible for necrosis of the islets and the consequent development of a diabetic syndrome. This, if confirmed, might have an important bearing upon the pathogenesis of spontaneous diabetes in man.

## SUMMARY

- 1 The observation of Shaw Dunn *et al* (1943) and others of the production of diabetes in the rabbit by alloxan has been confirmed.
2. In rabbits in which the main pancreatic duct has been ligated for 30 to 60 days pancreatic sclerosis with persistence of normal islet tissue is found.
- 3 In such animals alloxan has little or no pathological effect upon the islet tissue and does not produce diabetes.
- 4 Possible mechanisms for this effect are discussed.

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## ACTIONS OF CYANATE

BY

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The pharmacological actions of cyanate, except some recently described by one of us (Schutz 1945, 1946a), do not appear to have been previously investigated. This paper describes some of the general effects.

*Preparations*—Commercial preparations of sodium or potassium cyanate usually contain varying amounts of cyanide. No such contamination is present if cyanate is prepared from urea in one of the known ways (Haller, 1886a and b, Emich, 1889). Only preparations made from urea were used in the present investigation.

Ammonium cyanate was prepared from pure sodium cyanate (previously obtained from urea) through the silver salt. Silver cyanate, precipitated by mixing solutions of silver nitrate and sodium cyanate, was washed with water and acetone, a suspension of the dry salt in an aqueous solution of one equivalent of ammonium chloride was shaken for 1 hour. Silver cyanate has a very low solubility and the exchange into ammonium cyanate takes place slowly, leaving behind solid silver chloride. After 45–60 minutes the exchange is complete ( $\text{AgCNO} + \text{NH}_4\text{Cl} = \text{NH}_4\text{CNO} + \text{AgCl}$ ), and after centrifuging, the solution of ammonium cyanate was used within the next half hour. Solutions of ammonium cyanate are known to undergo the isomeric change into urea at an appreciable rate, even at room temperature. Solutions of sodium cyanate are, in comparison, much more stable, and were freshly made up each day.

*General effects in rats*—Doses of 5–15 mg/100 g sodium cyanate, injected intramuscularly, produced marked drowsiness after 10–15 minutes. If undisturbed the rats slept almost continuously, but during the sleep they could always be easily awakened. If suddenly aroused they sometimes exhibited a very short state of excitement, when they jumped once or twice, but they soon settled down again to sleep and complete immobility.

With higher doses (20 mg/100 g) some secretion from the eyes and nose was observed. With small doses (5–10 mg), which still produced marked drowsiness, no such increased secretion was observed. Even after very small doses (3–6 mg/100 g) the tendency to keep the eyes closed for some time was evident.

A graphical record of the activity of rats was made by a device which registers the movements of two suspended cages (Schutz, 1946b). Six rats were given cyanate and were placed in one cage and six similar controls, which were given water instead of cyanate, in another. A typical record is shown in Fig. 1.

The minimum effective dose which influenced the motility of rats (120–150 g) was found to be 2–3 mg/100 g. These doses produce a short but definite drowsiness. The effect of such small doses could, however, only be detected when the injections were given shortly before or during the normal time of wakefulness of the rat, i.e. after sunset, when the controls were invariably at the height of activity. In day time, when rats have their natural sleep periods, higher doses were required in order to produce a detectable difference, a significant difference could then be obtained after injection of 6–8 mg/100 g.

The drowsiness was marked but full anaesthesia could not be produced

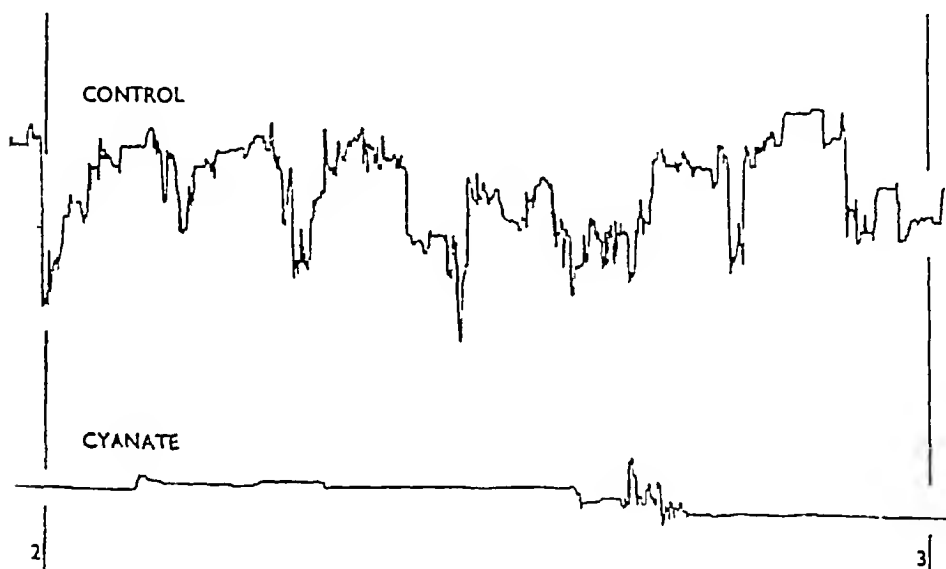


FIG 1—Motility record of 2 cages each containing 6 rats. The control (upper) record shows the activity characteristic for the peak period of wakefulness of rats during the early part of the night. The cyanate (lower) record shows the effect of 8 mg./100 g. sodium cyanate, given intramuscularly two hours before. Note the cessation of activity after cyanate, interrupted for a short period. Both records are for the third hour after the injections.

Although deeply asleep, the rats could always be aroused. As can be seen from the motility record shown in Fig 1, short periods of activity occur spontaneously between periods of profound quiescence. In this point the drowsiness caused by cyanate seems to differ essentially from the effect of any of the usual narcotic drugs, although the drowsiness lasts several hours after a dose of 10 mg/100 g. The threshold towards some stimuli did not seem to be much raised after small doses (5 mg/100 g), a sharp hiss or pinching of the tail usually eliciting the same reaction in the experimental and the control group.

Ammonium cyanate had apparently the same effect as sodium cyanate; an accurate comparison was difficult since the former is rapidly transformed into urea even at room temperature. In three experiments the drowsiness appeared

perhaps even more marked, though shorter in duration, after ammonium cyanate than after sodium cyanate

The drowsiness wore off completely, according to the dose given, e.g., 5-6 hours after 10 mg/100 g intramuscularly, 8-9 hours after 15 mg/100 g. After that period, and especially when higher doses were given, it was regularly noticed that the rats had not only recovered completely from the profound drowsiness, but also that they exhibited a certain increase in activity, although this could not be called a state of excitement. The rats which had received a large dose of cyanate the day before could always be recognized on the next day by their greater liveliness.

*Acute toxicity*—Although the actions of sodium cyanate are not strictly comparable with those of phenobarbitone, the LD 50 of each was determined simultaneously for female albino rats (100-150 g) of the same kind.

For sodium cyanate this was found to be  $31 (\pm 5)$  mg/100 g. body weight, for phenobarbitone, also injected intramuscularly,  $19 (\pm 6)$  mg/100 g. The figures ( $\pm$ s.d.) were obtained from a graphical estimate according to Gaddum's method (1933), using a group of 12 rats at three dose levels. The LD 50, expressed in gram molecules is thus for sodium cyanate  $4.76 \times 10^{-4}$ /100 g. for phenobarbitone  $8 \times 10^{-4}$ /100 g.

*Terminal convulsion*—If the lethal dose was in the region of 60-150 mg./100 g., death ensued with a terminal convulsion which was characteristic for cyanate. Non-lethal doses never caused convulsions in rats. The drowsiness gradually increased, the animals became apparently very weak and lay flat on the abdomen, without much support from the legs. The eyes were tightly shut and there were a few occasional movements, usually a short wiping of the nose and eyes with the forelegs. At the end of this period, a short convulsion was always seen. The animal very rarely recovered, but if it did, another convulsion occurred after an interval which might be several minutes, or as long as half an hour. No rat was seen to recover from this second convulsion.

Two stages of the convulsions could clearly be distinguished. The first stage started with the rat suddenly turning round and throwing itself either on its back or side. The head was maximally bent down and forwards, the nose and mouth being closely pressed against the breast. The eyes were tightly shut. From them and the mouth a small quantity of liquid was sometimes expelled. The lips were extremely retracted, producing a curious "grim-like" expression of the mouth, while the jaw seemed to be clenched. The ears were laid back. There was arrest of respiration. The forelegs were straight, extended, and maximally adducted to the abdomen. The paws were tightly bunched. The hindlegs were maximally stretched, the toes extended and fully splayed. Faeces were often expelled and, more rarely, urine. This stage only lasted 5-10 seconds. During the second stage many of the characteristics of the first one were altered in such a way that the ultimate death posture resulted. During this stage, clonic movements of apparently all the muscles occurred, while the head, ears, forelegs, and toes returned slowly to a more normal position, but the maximal extension of the hindlegs remained. During this stage, one could hear a deep inspiration. Sometimes the animal lay without any visible respiration, resuming its respiration gradually, but dying soon afterwards in another similar convulsion.

*Diuretic action*—Intramuscular injections of both sodium and ammonium cyanate were followed by a marked diuresis (Schütz, 1946a). This effect was

evident with rats and rabbits, even if no excess water was given previously. Typical results are shown in Fig. 2. For these experiments the rats were fully hydrated. They were given food and water up to the time of the injections. It can be seen that in the doses given, cyanate was at least as potent in rats as a number of other well-known diuretics. In order to investigate whether the diuretic effect of injected cyanate could be ascribed to ammonia, which may be formed from cyanate in the body, an equivalent amount of ammonia was injected and found to be ineffective. For the same reason the action of urea, cyanide, and thiocyanate was recorded with similar animals.

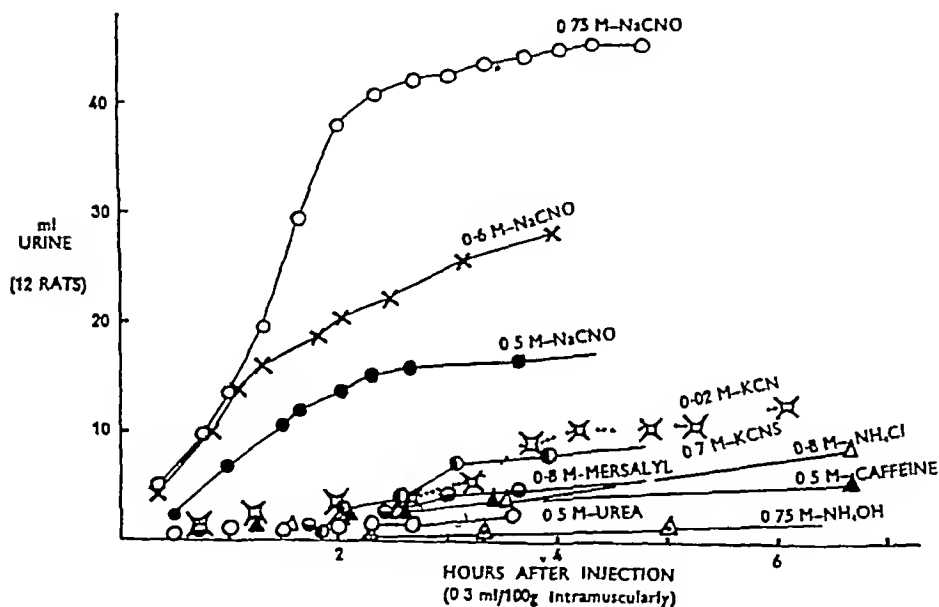


FIG 2—Diuretic action of cyanate. Doses 0.3 ml./100 g. No excess water was given previously.

From the evidence submitted in Fig. 2 it can be concluded that the diuretic action must be due to the CNO group, and not to an eventual breakdown or transformation product. Ammonia was quite inactive, the diuretic potency of urea was found to be approximately a thirtieth of that of cyanate. Cyanide could not, for obvious reasons, be given in the same doses. Fig. 2 shows the diuretic effect of a dose of KCN which is approximately 50 per cent of a dose found to be lethal in 10 rats.

**Blood sugar**—Intravenous injection of sodium cyanate in rabbits was followed by a similar, but less obvious, drowsiness to that observed in rats. A small and transient rise of the blood sugar was recorded with doses from 5 mg./100 g upwards. Typical results are shown in Table I.

TABLE I

SMALL AND TRANSIENT RISE OF BLOOD SUGAR IN RABBITS AFTER INTRAVENOUS INJECTION OF SODIUM CYANATE.

Dose, in mg./100 g body weight	Blood sugar in mg./100 ml at stated intervals of time after the injection					
	30'	60'	90'	120	150	24 hr
5 mg. NaCNO	105	145	160	148	120	112
9 mg NaCNO	112	165	170	140	115	110
Saline	95	112	106	95	95	95
Saline	95	110	102	100	105	105

Even with the highest tolerated doses only a transient rise was observed. Also after daily intramuscular injections of 6 mg /100 g over a period of 10 days the blood sugar was found to be within normal limits 24 hours after the last injection (80-90 mg /100 ml, in 6 rabbits, not starved). The observed levels were always rather low, probably owing to the much reduced intake of food mentioned below.

*Body temperature*—Intravenous and intramuscular injections in rabbits were followed by a significant fall in body temperature. The rabbits were starved 12 hours before the injections. The effect was taken as the lowest rectal temperature recorded within 3 hours of the injection. The mean value in 6 rabbits receiving saline on three occasions was  $38.9^{\circ} \pm 0.2$  (S.D. mean) while that of 6 rabbits receiving sodium cyanate in three experiments was  $37.7 \pm 0.3$ . There was an interval of 3-4 days between the experiments. The drug thus caused a clearly significant mean fall of temperature of  $1.2^{\circ} \pm 0.36$  ( $P < 0.1$ ) compared with the controls. At 24 hours after the last injection the mean temperature of 6 rabbits receiving sodium cyanate in two experiments was  $38.8 \pm 0.34$  and that of 6 control rabbits receiving saline by the same route in two experiments was  $38.7 \pm 0.31$ . The temperature was thus in the normal range and not significantly different from that of the control rabbits.

*Effects of repeated injections*—While the effects of a single injection of even a large dose of cyanate (10 mg /100 g.) wore off completely, smaller doses repeated daily soon caused a continuous drowsiness in rats and rabbits, only interrupted for eating and drinking. This was very marked from the third or fourth injection onwards. Similarly, the effect of smaller doses seemed to increase. Of 24 rats which were given 6 mg /100 g daily two died within 2 weeks. Soon after the injections were stopped no abnormality in the behaviour of the rats could be observed. No withdrawal symptoms were noted. During the period of the injections the rats rarely moved at all, except to take food and drink. They lost weight and ate less, but drank more and had periods of diarrhoea. At the end

of this period their body weight was on average 34 per cent below that of the controls which were given daily injections of saline

Daily intramuscular injections of 10 mg/100 g in rabbits produced an increasing drowsiness and apathy. They took very little food although when aroused they reacted very similarly to the controls. They lost weight rapidly and appeared so emaciated that from the fourth injection the dose was reduced to 7.5 mg/100 g and from the sixth injection to 5 mg/100 g. Eight rabbits were injected in this way for 18 days. One died on the seventh day. The others recovered completely and soon gained weight. Their blood sugar was within normal limits

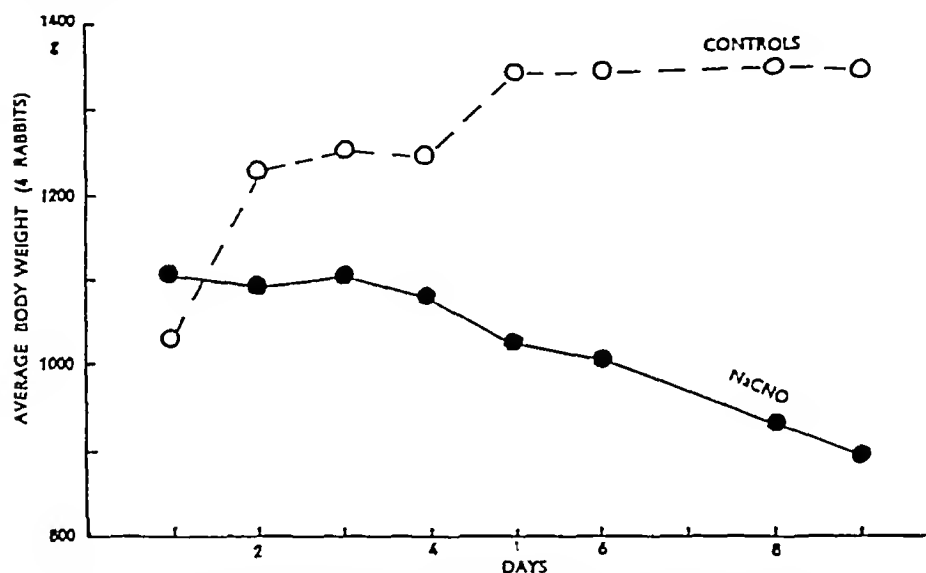


FIG 3—Fall of average body weight of 4 rabbits during a period of daily injections of sodium cyanate. Average weight of 4 saline-treated controls is also shown

The average weight of two groups of 4 rabbits is shown in Fig. 3. One group received daily intramuscular injections of sodium cyanate (10 mg./100 g.) and the control group received the same volume of saline

The faeces of both rats and rabbits usually became very moist in consistency. This moistness however, often disappeared, although the dose was not changed. Sometimes the faeces were found to be much paler in colour than those of the controls. This symptom, which was not regularly seen, disappeared without any change in the daily dosage.

Rabbits and guinea-pigs which were injected with doses causing death after 6–8 days showed an almost empty intestinal tract. No formed faeces were found and there was hardly any visible fat in those rabbits which received the injections for at least one week. The heart was always found distended and full of blood. A definite hyperaemia was observed in the lower pelvic region. No other obvious abnormality was found. The absorption spectrum of the blood of these animals showed no difference from that of normals as observed with the Hartridge reversion spectrocope.



Although no determination of the L.D. 50 in rabbits and guinea-pigs was attempted, the few data obtained with these animals suggested that the toxicity of sodium cyanate given intramuscularly was of the same order of magnitude for rabbits, guinea-pigs, and rats. On repeated injections rabbits seemed to be more sensitive than rats.

*Comparison with cyanide*—Although injected cyanate is probably transformed into urea in the body, the possibility could not be excluded *a priori* that cyanate might be reduced to cyanide in the body. After repeated injections of cyanate into three rabbits, with a lethal dose as the last injection, no trace of cyanide could be found in their blood and collected organs. The same method of detection (Feigl, 1943) gave a strongly positive test for cyanide in the blood and organs of a rabbit which received a minimum lethal dose of cyanide. When the actions of cyanide were compared with those of cyanate in similar rats a number of differences were observed. After injection of cyanide, rats became dull and lifeless and moved very little, but they did not appear to have the long and deep sleep which was produced by medium doses of cyanate. The cyanide rats usually had their eyes wide open and the drowsiness never lasted longer than  $\frac{1}{2}$ –1 hour. A further striking difference between cyanate and cyanide was the terminal convulsion which was produced after the former drug, and the death posture regularly found after it. Cyanide, as well as cyanate, had a marked diuretic action. Compared with its toxicity, cyanide was, however, much less active than cyanate.

## DISCUSSION

The main actions of cyanate, as observed in the whole animal, which are reported in this paper, were compared with those which can be produced by ammonia, urea, cyanide, and thiocyanate. All these comparisons, as well as the identical action obtained with pure preparations of sodium and ammonium cyanate made from urea, support the view that the actions are due to the CNO group and not to any of the above-mentioned substances into which injected cyanate might conceivably be transformed in the body.

## SUMMARY

- 1 The general effects of injected sodium and ammonium cyanate on the intact animal were studied.
- 2 Cyanate produces pronounced drowsiness and sleep in rats, but not full anaesthesia.
- 3 Lethal doses produce a characteristic terminal convulsion and death posture in rats.
- 4 Intramuscular injections of cyanate produce a marked diuresis and diarrhoea in rats and rabbits.

5 There is a small rise of blood sugar and a small fall of body temperature after the injection of cyanate into rabbits Both changes are transient

6 Repeated daily injections of cyanate lead to a rapid fall in body weight Blood sugar and body temperature remain within normal limits

7 It is concluded that the actions are due to the CNO group and not to any substance into which injected cyanate may conceivably be transformed in the body (urea ammonia, cyanide, or thiocyanate)

We are greatly indebted to Mr Garfield Thomas for having determined the blood sugar levels mentioned in this paper and to Dr R F A Dean and Professor A C Frazer for help given in the presentation of our results Thanks are also due to Glaxo Laboratories for presenting us with a specially prepared sample of pure sodium cyanate, and to John Tatlow for technical assistance

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# THE ACTION OF MERSALYL, CALOMEL AND THEOPHYLLINE SODIUM ACETATE ON THE KIDNEY OF THE RAT

BY

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It has been shown that the creatinine clearance in man (Blumgart, Gilligan, Levy, Brown, and Volk, 1934) or dogs (Davenport, Fulton, Van Auken and Parsons, 1934, Walker, Schmidt, Elsom, and Johnston, 1937) is not increased by mercurial compounds, nor did thermostromuhr measurements in anaesthetized dogs reveal any constant changes of the renal blood flow when mercurial diuretics were injected (Walker *et al*, 1937). It was, therefore, concluded that the mercurial compounds exert their diuretic effect by reducing the tubular water reabsorption. Objections could be raised against the interpretation of some of these experiments: (1) the creatinine clearance can no longer be regarded as the expression of the glomerular filtration rate in man (Shannon, 1935), (2) anaesthetics or surgical measures like those necessary for thermostromuhr measurements are likely to have a disturbing effect on renal function (Smith, 1943). It was, therefore, decided to reinvestigate the renal action of mercurial compounds in unanaesthetized intact animals by means of simultaneous inulin and diodone clearance estimations. The albino rat was chosen because it had previously been shown (Dicker and Heller, 1945) that renal function in this species resembles that of dog and man.

## METHODS

*Experimental animals*—Male adult albino rats of an average weight of 176.5 g. were used throughout. The animals were kept on a standard diet (vitamin A test diet, U.S.P., XI revised 1937, with the addition of cod liver oil and of tocopherol) for some time before the experiments were done.

*Experimental procedure*—The experimental procedures for the determination of simultaneous inulin and diodone clearances in rats have been described in a previous paper

(Dicker and Heller 1945) Inulin in plasma and urine was determined by the method of Smith, Goldring and Chasis (1938) and diiodone iodine in plasma and urine by that of Alpert (1941). Chloride in plasma was estimated by Whitehorn's method (1921) chloride in urine by that of Volhard Arnold Chlorides were expressed in terms of NaCl Inulin (Kerfoot & Co) and Per Abrodil (Bayer Products Ltd) were used

*Diuretic drugs used*—(1) Mersalyl (Burroughs Wellcome & Co London) is described by the firm of makers as "a complex mercurial compound containing about 40% of the metal in a non-ionizable form" It contains also 5% of theophylline sodium acetate

(2) Mercurous chloride (calomel) The preparation used was a 0.14% suspension of mercurous chloride in adeps lanae and oil arachidis

(3) Theophylline sodium acetate

*Mode of administration of the diuretics*—Mersalyl and calomel were injected intramuscularly Theophylline sodium acetate was administered either by stomach tube or by intramuscular injection

*Methods for quantitative comparison of diuretic activity*—During the period of experimentation the animals were kept in individual glass metabolism cages as previously described (Dicker and Heller, 1945) In the experiments with mersalyl calomel and theophylline sodium acetate the rats were given 5% of their body weight of tap water by stomach tube The action of these drugs on the rate of urine flow was compared with that of non-injected control rats which received the same amount of water The volume of urine was measured at intervals of 30 minutes, during a total period of 3 hours and expressed as percentage of the volume of water given

*Evaluation of clearance estimations*—The nomenclature and method of calculation of glomerular filtration rate (GFR) effective renal plasma flow (RPF) and total tubular excretory mass ( $Tm_D$ ) follow substantially those adopted in the publications of Smith (1943) and his associates The rate of tubular reabsorption of chloride ( $T_{Cl}$ ) was calculated as follows  $T_{Cl} = (P_{Cl} \times C_{In}) - (U_{Cl} \times V)$  where  $P_{Cl}$  = concentration of plasma chloride in mg./100 ml  $C_{In}$  = inulin clearance in ml/100 g./min  $U_{Cl}$  = concentration of urinary chloride in mg./100 ml., and  $V$  = urine flow in ml/min The rate of the tubular water reabsorption ( $T_W$ ) was calculated as  $T_W = C_{In} - V$  In order to permit the comparison of  $T_W$  and  $T_{Cl}$  values obtained at different values of GFR,  $T_W$  and  $T_C$  were regularly expressed as the percentage of water and chloride filtered

*Statistical treatment of results*—Fisher's "t" test was applied for estimation of the significance of the difference of means "Small sample" methods were used for populations less than 15 The correlation coefficient "r" and the interclass coefficient "z" were calculated according to Mainland (1938) The probability (P) for "t" or "r" was obtained from Fisher and Yates (1943) tables

## RESULTS

### 1 Normal rats

To obtain a basis of comparison for the results of the experiments with diuretic compounds values for the different renal functions in normal control rats have first to be given

Normal rats which received 5% of their body weight of water excreted in 180 min  $81.5 \pm 2.66\%$  (S.E. of mean of 108 observations) of the amount of water given (Fig. 1) expressed in terms of ml/100 g body weight/min they excreted

during these three hours an average of  $0.023 \pm 0.0004$  ml / 100 g / min of urine, with a mean sodium chloride concentration of  $0.8 \pm 0.08$  mg / 100 g / min

The renal function tests during such a water diuresis showed that the glomerular filtration rate (GFR), the renal plasma flow (RPF), the filtration fraction (FF), the total tubular excretory mass ( $Tm_p$ ), and the rate of tubular chloride reabsorption ( $T_c$ ) remained practically unchanged for urine flows ranging from 0.0017 to 0.1030 ml / 100 g / min. The mean GFR amounted to  $0.35 \pm 0.005$  (134) ml / 100 g / min and the mean RPF to  $2.22 \pm 0.006$  (28) ml / 100 g / min, the mean FF was  $0.17 \pm 0.003$  (28), the mean  $Tm_p = 0.13 \pm 0.002$  (84) mg / 100 g / min, and the mean  $T_c = 97.1 \pm 0.25\%$  (41). Figures in parentheses indicate the number of observations.

In contrast to the stability of these different functions, the rate of tubular water reabsorption ( $T_w$ ) showed a decrease closely correlated with the increase of the rate of urine flow ( $r = -0.99$ , S.E. =  $\pm 0.089$ ,  $P < 0.001$  (134)).

It can therefore be concluded that a water diuresis in a normal rat is solely effected by changes in the rate of tubular water reabsorption.

## II Renal effects of mersalyl

Two series of experiments were performed, in the first, rats were injected with a low, non-diuretic amount of mersalyl ( $0.0006$  mM / 100 g), in the second, the animals were injected with  $0.0027$  mM / 100 g mersalyl. This last dose has been shown to exert the optimum diuretic effect in rats (Lipschitz, Hadidian, and Kerpcsar, 1943).

(1) *Effect of 0.0006 mM / 100 g mersalyl* After injection with mersalyl, the rats were divided into three groups: (i) those which received 5% of their body weight of water after one hour, (ii) those which were given water 2 hours after the injection, (iii) those to which the standard dose of water was given after 10 hours. The rate of urine flow of each group was measured during 3 hours following the administration of water and the quantity excreted expressed in percentage of the water given. No significant difference could be noted between the rate of urinary excretion in any of these rats and that of normal controls which had received the same standard amount of water.

To ascertain whether a non-diuretic amount of mersalyl had any effect on the kidneys at all, inulin and diodone clearance determinations were carried out in the three groups of rats, inulin and diodone being injected about 1 hour before water was given. The results were comparable in the three groups and can be considered together (Table I). The mean value for GFR, RPF, and FF were significantly increased (t for the respective values was found to be 11.43,  $P < 0.001$ , 7.50,  $P < 0.001$ , and 6.89,  $P < 0.001$ ).

Tubular activity in these animals as gauged by the rate of chloride reabsorption and the maximum rate of transfer of diodone did not differ significantly from

that of the controls (Table I), (t for the respective values was found to be 1.58,  $P>0.1$ , and 0.36,  $P>0.7$ ) The rate of tubular water reabsorption was, as in control rats, significantly correlated with the rate of urine flow ( $r = -0.94$ ,  $S.E. = \pm 0.258$ ,  $P<0.001$ )

TABLE I

RENAL EFFECTS OF MERSALYL, CALOMEL, THEOPHYLLINE SODIUM ACETATE  
AND A HYPOTONIC NaCl SOLUTION

The figures are mean results with their standard error  $r \pm S.E.$  = correlation coefficient between the value indicated and the urine flow, and standard error of  $r$  Number of experiments in parentheses

	GFR (Inulin Clearance) ml /100 g./min		RPF (Diodone Clearance) ml /100 g./min		$T_{mH_2O}$ (mg l./100 g./min) $M \pm S.E.$	$T_{Cl}$ (as % of chloride filtered) $M \pm S.E.$	$T_{H_2O}$ (as % of water filtered) $r \pm S.E.$
	$M \pm S.E.$	$r \pm S.E.$	$M \pm S.E.$	$r \pm S.E.$			
Control rats (134)	0.35 $\pm 0.005$	—	2.22 $\pm 0.006$	—	0.13 $\pm 0.002$	97.1 $\pm 0.25$	-0.99 $\pm 0.089$
Mersalyl 0.0006 mM /100 g. (18)	1.11 $\pm 0.075$	—	3.62 $\pm 0.190$	—	0.13 $\pm 0.008$	97.8 $\pm 0.39$	-0.94 $\pm 0.258$
Mersalyl 0.0027 mM /100 g. (20)	1.02 $\pm 0.067$	+0.79 $\pm 0.223$	3.51 $\pm 0.486$	+0.86 $\pm 0.392$	0.035 $\pm 0.0098$	98.4 $\pm 0.28$	-0.97 $\pm 0.223$
Calomel 0.00027 mM /100 g. (18)	1.04 $\pm 0.176$	+0.75 $\pm 0.360$	3.42 $\pm 0.578$	—	0.13 $\pm 0.011$	99.5 $\pm 0.07$	-0.54 $\pm 0.360$
Theophylline 0.01 mM /100 g. (19)	1.05 $\pm 0.109$	+0.72 $\pm 0.330$	4.15 $\pm 0.342$	+0.71 $\pm 0.313$	0.13 $\pm 0.083$	98.6 $\pm 0.29$	-0.68 $\pm 0.302$
NaCl 85 mM /100 g. (22)	1.08 $\pm 0.035$	—	2.72 $\pm 0.154$	—	0.14 $\pm 0.009$	98.2 $\pm 0.18$	-0.90 $\pm 0.210$

It can therefore be assumed that the renal effect of a non-diuretic dose of mersalyl is mainly characterized by a vascular reaction, without any appreciable changes in the tubular activity

(2) *Effect of 0.0027 mM /100 g mersalyl* Three groups of well hydrated rats were injected with 0.0027 mM /100 g mersalyl The standard amount of water was given 2, 4, and 10 hours after the administration of the diuretic The urinary excretion was measured for 3 hours, and expressed as percentage of the water given No diuretic action could be noticed in the two first groups, i.e., in rats which received 5% of their body weight of water 2 and 4 hours after the

injection of mersalyl. But a clear diuretic effect could be observed in the rats to which the standard amount of water was given 10 hours after the mercurial compound. In that group the mean average excretion of urine observed during 3 hours was  $0.029 \pm 0.0041$  (18) ml./100 g./min (t with control rats = 2.46,

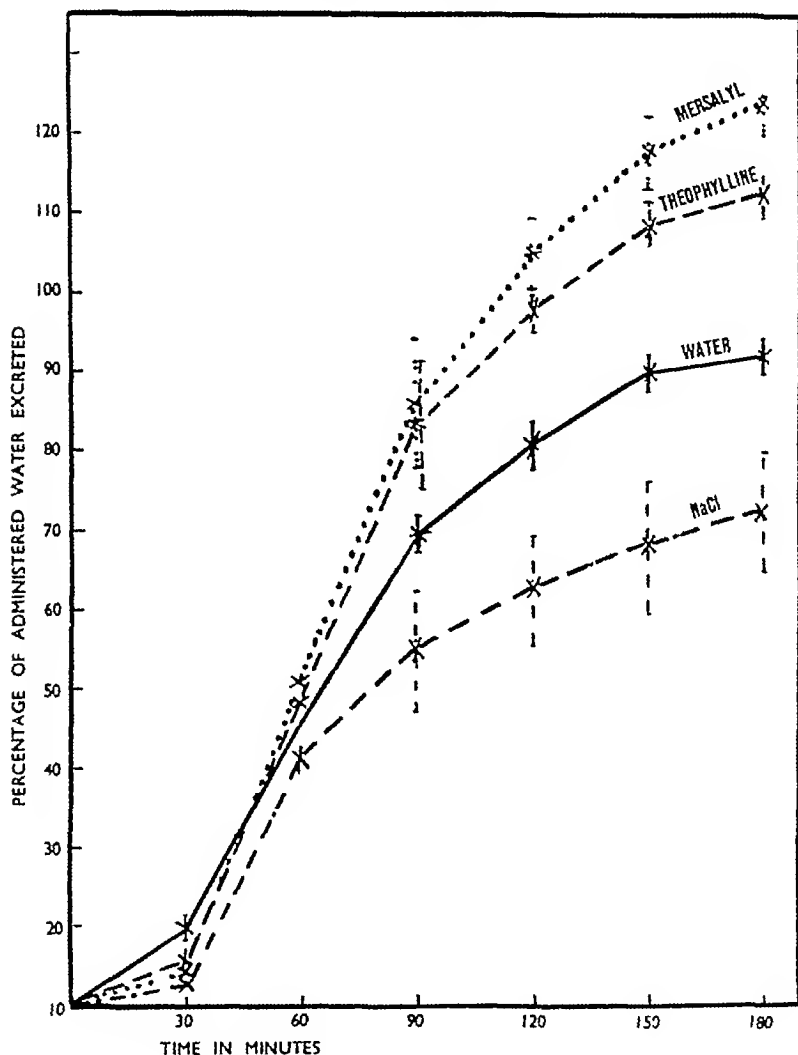


FIG 1.—Effect of diuretics on the water diuresis of rats. All rats received 5% of their body weight of water by stomach tube.  $\times$ — $\times$  controls (water only)  $\times$ — $\times$  rats injected with 0.0027 mM / 100 g mersalyl  $\times$ — $\times$  rats injected with 0.01 mM / 100 g theophylline sodium acetate  $\times$ — $\times$  rats which received 5.0 ml / 100 g of an 85 mM NaCl solution by stomach tube. The vertical lines indicate the standard error.

$P < 0.02$ ) with an average sodium chloride concentration of  $4.5 \pm 0.31$  mg / 100 g / min ( $t = 11.34$ ,  $P < 0.001$ ) Fig 1 shows the amount of urine excreted during that period, expressed in percentage of the dose of water given, after 180 minutes the "mersalyl rats" excreted  $114.0 \pm 5.55\%$  (18) compared with  $81.5 \pm 2.66\%$  ( $t = 5.66$ ,  $P < 0.001$ ) in the controls. It should be pointed out that in no instance had the mercurial any diuretic effect when water was not supplied to the animals, a fact which agrees with the findings of Fournneau and Melville (1931) and of Sollman and Schreiber (1936).

To determine the renal mechanism responsible for the enhanced diuresis observed in the last group inulin and diodone were injected about 1 hour before the water was given, i.e., 9 hours after the administration of  $0.0027$  mM / 100 g mersalyl. Clearance determinations were carried out in the usual way. Most of these rats were found to have sugar in their urine and glucose clearances were therefore estimated, whenever possible, simultaneously with the others. It should be noted that, in nearly all these experiments, an appreciable amount of albumin could be detected.

The mean value for GFR in this series was much the same as that observed after the injection of  $0.0006$  mM / 100 g mersalyl (mean GFR =  $1.02 \pm 0.067$  (20),  $t = 0.86$ ,  $P > 0.4$ ), but in contrast to the latter was found to be correlated with the urine flow ( $r = +0.79$ ,  $SE = \pm 0.223$ ,  $P < 0.001$ ) and with the renal plasma flow ( $r = +0.83$ ,  $SE = \pm 0.267$ ,  $P < 0.001$ ).

The increase of GFR could be explained by a change in the permeability of the glomerular vessels (albuminuria) were it not for the fact that the rate of glomerular filtration is significantly correlated with both the urine flow and the renal plasma flow. It seems, therefore, more likely that the mercurial compound produced, directly or indirectly, an increase in the blood supply, which, in turn, would increase the rate of glomerular filtration. It may in any case be assumed that the enhanced diuresis observed in rats injected with  $0.0027$  mM / 100 g. mersalyl is to some extent a function of the increased rate at which water is filtered through the glomeruli.

The reabsorption of chloride was also found to be enhanced. The values for  $T_{Cl}$  were not correlated with the increased GFR or the urine flow. The mean value for  $T_{Cl}$  was found to be  $98.4 \pm 0.28\%$  (20), which is significantly higher than that observed in control rats ( $t = 3.54$ ,  $P < 0.001$ ). It can therefore be concluded that in "mersalyl rats" chloride is reabsorbed at a higher rate than in normal animals. But how can this fact be reconciled with the increased concentration of urinary chloride? When the mean plasma chloride concentration was compared with that observed in control rats, it was found that, whereas the mean plasma chloride for normal rats was  $356.2 \pm 18.40$  (41) mg / 100 ml, the mean plasma chloride in rats injected with  $0.0027$  mM / 100 g mersalyl was  $430.5 \pm 8.68$  (20) mg / 100 ml, which is significantly different ( $t = 3.45$ ,  $P < 0.001$ ). This fact agrees with the findings of Crawford and McIntosh (1925), who showed that



after injection of novasurol there was a transfer of chlorides from tissues to plasma. If more chloride is filtered because the plasma chloride concentration is higher or because the glomerular filtration rate is increased, or because both these factors apply, more chloride should be excreted, unless the excess of chloride filtered is reabsorbed by the tubules.

It seems likely that mersalyl has some depressor effect on tubular activity. This is suggested by the markedly decreased  $Tm_D$  (mean  $Tm_D = 0.035 \pm 0.0098$  mg/100 g/min, as compared with  $0.13 \pm 0.002$  in control rats,  $t = 9.74$ ,  $P < 0.001$ ) and by the fact that glucose was found in the urine of these rats. The objection may be made that changes in the rate of filtration and hence in the amount of glucose delivered to the tubules may produce glycosuria (Shannon, 1938) without any decrease in the capacity for glucose reabsorption. However, glycosuria after mersalyl has been observed at low as well as at high glomerular filtration rates, i.e., in circumstances in which the amount of sugar delivered to the tubules was very low in one instance and very high in another.

### *III Renal effects of mercurous chloride*

Various doses of mercurous chloride (calomel) were tried. The dose of 0.0027 mM/100 g, approximately equivalent to the mercury content of the higher dose of mersalyl used, proved to be toxic, so was the dose of 0.00135 mM/100 g calomel, though to a lesser degree. Rats injected with these doses remained in a state of anuria for several hours and developed severe oedema, with large amounts of free fluid in the abdominal cavity. The scanty urine finally passed was regularly mixed with blood. After several trials the dose of 0.00027 mM/100 g rat was adopted. This dose has been shown to be highly diuretic in rabbits (Fourneau and Melville, 1931).

(a) *Effect of injections of calomel (0.00027 mM/100 g rat) on the diuresis*  
The standard amount of water was given by stomach tube 2, 3, or 10 hours after the injection of calomel, the urine was measured every 30 minutes during 3 hours after water administration. The amounts of urine excreted were expressed, as usual, in percentage of the amount of water given. In none of these groups did calomel exert a diuretic effect, the total quantity of urine excreted in 3 hours was  $82.8 \pm 2.47\%$  (48) of the standard amount of water given, a figure which is statistically comparable with that obtained in control rats which had received the same amount of water ( $t = 0.36$ ,  $P > 0.7$ ), but the urine excreted during these 3 hours contained a much higher concentration of sodium chloride than that of normal animals  $1.7 \pm 0.30$  mg/100 g/min, as compared with  $0.8 \pm 0.08$  mg/100 g/min ( $t = 2.93$ ,  $P < 0.01$ ), but it contained significantly less than that of "mersalyl rats" ( $t = 6.43$ ,  $P < 0.001$ ).

(b) *Renal effects of 0.00027 mM/100 g calomel*  
The glomerular filtration rate of rats injected with 0.00027 mM/100 g of calomel was increased (mean

GFR =  $1.04 \pm 0.176$  (17) ml / 100 g / min ) and correlated with the rate of urine flow ( $r = +0.75$ ,  $SE = \pm 0.360$   $P < 0.001$ ) The renal plasma flow was also increased (mean RPF =  $3.42 \pm 0.578$  (7) ml / 100 g / min ), but not correlated with the urine flow

Concerning the tubular activity, it will be seen (Table I) that the tubular excretory mass ( $Tm_D$ ) compared with that of control rats was not affected (mean  $Tm_D = 0.13 \pm 0.011$  (10) mg / 100 g / min ,  $t = 0.24$ ,  $P > 0.9$ ), but that the tubular rate of chloride reabsorption was significantly increased (mean  $T_{Cl} = 99.5 \pm 0.07\%$  (17),  $t = 3.11$ ,  $P < 0.001$ ) and comparable to that of rats injected with 0.0027 mM / 100 g mersalyl ( $t = 1.42$ ,  $P > 0.2$ ) It will also be noted that as in rats injected with mersalyl, the plasma chloride concentration was significantly higher than that of control rats ( $t = 2.76$ ,  $P < 0.001$ ) The increased rate of chloride reabsorption was accompanied by an increase in the rate of tubular water reabsorption and the correlation coefficient between  $T_w$  and rate of urine flow lost its significance ( $r = -0.54$ ,  $SE = \pm 0.360$ ) In other words the rate of water reabsorption, expressed as percentage of the water filtered ( $T_w$ ), remained practically the same at any rate of urine flow, a finding which is in sharp contrast to that described for normal rats

It has been shown that, in contrast to mersalyl, calomel did not exert any diuretic effect. The mercurial compounds had the same effect on the glomerular filtration rate in both series, and the correlation coefficients between GFR and rate of urine flow were almost the same (mean  $z = 0.08$ ,  $SE = \pm 0.595$ ) The diuretic effect of mersalyl and the non-diuretic action of calomel were thus due to the difference between the mean rates of tubular water reabsorption

The fact that the plasma chloride concentration was found to be higher in rats injected with mersalyl or with calomel than in control rats, and the fact that the rate of chloride filtration was also higher, raises the question whether the renal effect of these mercuric compounds is not partly the result of an increase in the osmotic pressure of the fluid passing through the glomeruli

To answer this question, rats were given 5% of their body weight of 85 mM NaCl solution by stomach tube and the diuresis and renal function were investigated in the usual way

The mean diuresis of these rats is recorded in Fig 1 and shows a decrease in the urine flow The results of the clearance tests showed normal values for the total tubular excretory mass (mean  $Tm_D = 0.143 \pm 0.0093$  mg / 100 g / min.), but values of  $T_{Cl}$  of the same order as those observed in rats injected with 0.0027 mM / 100 g mersalyl (mean  $T_{Cl} = 98.2 \pm 0.18$ ,  $t = 0.46$ ,  $P > 0.6$ ) The mean plasma chloride concentration was  $486.4 \pm 7.87$  mg./100 ml , it was thus slightly higher than that of the rats injected with mersalyl The increase observed in the glomerular filtration rate (mean GFR =  $1.08 \pm 0.035$  (22) ml./100 g./min ) was partly produced by an increase in the filtration fraction (mean FF =  $0.41 \pm 0.032$ ) and partly by an increase in the renal plasma flow (mean RPF = 2.72

$\pm 0.154$  ml / 100 g / min ), but GFR was not correlated either with the rate of urine flow or with the rate of tubular water reabsorption. When compared with the mean GFR in rats injected with mersalyl, the glomerular filtration rate proved to be of the same magnitude ( $t=0.79$ ,  $P>0.4$ ). It may therefore be assumed that the chloride concentration of the glomerular filtrate in the rats which received a saline solution (85 mM NaCl) was much the same as that of the glomerular filtrate in rats injected with mersalyl. However, far from having a pronounced diuretic effect, like that of mersalyl, an 85 mM NaCl solution lowered the rate of diuresis.

Comparison of the coefficient of correlation between  $T_w$  and rate of urine flow in rats which received 5% of their body weight of an 85 mM. NaCl solution with that of rats injected with mersalyl, will show that  $r = -0.90$ ,  $P<0.001$ , in the former, but that  $r = -0.97$ ,  $P<0.001$ , in the latter. A statistical comparison revealed a significant difference (mean  $z=1.78$ ,  $S.E. = \pm 0.166$ ) between these values. It is thus evident that the plasma chloride concentration and the glomerular filtration rate in rats which received a hypotonic NaCl solution was increased to much the same degree as in rats injected with mersalyl. However, the former reabsorbed more water per unit time than the latter. It would seem therefore that the increase in the diuresis observed in rats injected with mersalyl cannot be explained by the increased amount of chloride filtered, but must be explained by some other action of the mercurial compound.

#### IV Renal effects of theophylline sodium acetate

There is a widespread opinion that xanthine derivatives induce hyperaemia in the mammalian kidney (Cushny, 1917, Richards and Plant, 1922, Janssen and Rein, 1928, Ellinger, 1929, Verney and Winton, 1930, Walker, Schmidt, Elsom, and Johnston, 1937). However, Chasis, Ranges, Goldring, and Smith (1938) examined the action of theophylline on man by means of simultaneous inulin and diodone clearances and concluded that theophylline and caffeine consistently reduced the blood flow through the normal human kidney, but increased the glomerular filtration rate and the filtration fraction.

To investigate the diuretic action of theophylline in rats the drug was injected subcutaneously or given by mouth, as suggested by Lipschitz *et al* (1943). These investigators have shown that within certain limits there is a linear relationship between log dose of theophylline and log action in the rat, but that an overdose produces a decrease in the diuretic effect. They established 0.07 and 0.10 mM / kg. as the optimum diuretic dose for the rat. A dose of 0.01 mM / 100 g theophylline was, therefore, chosen and given by mouth in the present series.

The diuretic effect of 0.01 mM / 100 g rat given in 5% of body weight of water by stomach tube was first investigated. In two groups of rats the urinary output was measured for 3 hours and 10 hours respectively. The rats whose

diuresis had been measured during the longer period received a second dose of water 10 hours after the first to ascertain whether the diuretic effect of the theophylline had ceased

In the first group, theophylline exerted a marked diuretic effect the total amount of urine excreted in 3 hours exceeding the quantity of water given by nearly 5% (Fig 1) When comparing the total urinary chloride excretion in this group with that of control rats, it was found that rats which had received a theophylline solution excreted  $2.2 \pm 0.23$  mg NaCl/100 g/min the control rats excreting only an average of  $0.8 \pm 0.08$  mg NaCl/100 g/min ( $t=5.78$ ,  $P<0.001$ )

In the second group i.e., in the rats whose urine was collected for 10 hours, a comparison with control rats showed that "theophylline rats" excreted  $0.012 \pm 0.0011$  (24) ml/100 g/min with a mean chloride content of  $2.1 \pm 0.61$  mg/100 g/min NaCl while in the control rats the urine excretion during 10 hours amounted to  $0.009 \pm 0.001$  ml/100 g/min and a mean chloride content of  $1.4 \pm 0.36$  mg/100 g/min The mean quantity of urine excreted by the rats which received theophylline was significantly higher than in control rats  $t=2.65$ ,  $P<0.05>0.02$  but the difference between the mean chloride concentrations was not significant  $t=0.97$ ,  $P>0.4$  However, if after 10 hours "theophylline rats" and controls were given 5% of their body weight of water, the water diuresis was found to be the same in the two groups ( $t=0.04$ ,  $P>0.9$ ), the chloride excretion being also comparable in the two groups ( $t=0.63$ ,  $P>0.5$ )

It can therefore be concluded that theophylline sodium acetate given by mouth, simultaneously with 50 ml/100 g water, produces a marked increase in the urine flow and in the chloride excretion Compared with a dose of 0.0027 mM/100 g mersalyl, these effects of theophylline were of comparatively short duration, after 10 hours, and possibly sooner, the water diuresis and the chloride excretion had returned to normal values

To investigate the renal mechanism of the xanthine diuresis, rats were given theophylline either by stomach tube or by subcutaneous injections simultaneously with 50 ml/100 g of water Inulin and diodone clearances were carried out in the usual way

The main features of the results of the clearances determinations were (a) an increase in both glomerular filtration rate (mean GFR= $1.05 \pm 0.108$  (24) ml/100 g/min) and renal plasma flow (mean RPF= $4.15 \pm 0.342$  ml/100 g/min), (b) a significant correlation between GFR and rate of urine flow ( $r=+0.72$ ,  $SE=\pm 0.330$ ,  $P<0.001$ ), (c) a significant correlation between RPF and GFR ( $r=+0.68$ ,  $SE=\pm 0.313$ ,  $P<0.001$ ) and between RPF and rate of urine flow ( $r=+0.71$ ,  $SE=\pm 0.313$ ,  $P<0.001$ ) The difference between these three coefficients of correlation is not significant, as can be shown by transforming the values of "r" into the interclass coefficient "z."

The total tubular excretory mass ( $Tm_D$ ), throughout the whole range of

urine flow remained unchanged and was of normal magnitude (mean  $T_{mD} = 0.13 \pm 0.083$  (14) mL/100 g/min). However, an increase of the filtration fraction was noted (mean  $FF = 0.28 \pm 0.035$ , compared with  $0.17 \pm 0.002$  in control rats,  $t = 3.29$ ,  $P < 0.001$ ), which suggests a slight constriction of the glomerular efferent vessels.

The data in Table I show that in theophylline diuresis the rate of tubular water reabsorption, as expressed in percentage of the glomerular filtration rate, was not as closely correlated with the rate of urine flow as in control rats (coefficient of correlation between  $T_w$  and the rate of urine flow  $r = -0.68$ ,  $S.E. = \pm 0.302$ ,  $P < 0.001$ , compared with  $r = -0.99$  in control rats).

$T_{Cl}$  remained practically constant at both low and high rates of urine flow encountered in these experiments (mean  $T_{Cl} = 98.6 \pm 0.29\%$ ). It follows that, compared with the rate of tubular chloride reabsorption in control rats, theophylline increased the rate at which chloride was reabsorbed ( $t = 3.99$ ,  $P < 0.001$ ).

A comparison of the renal mechanism of diuresis in "theophylline rats" and in controls leads to the conclusion that the rate of glomerular filtration takes an active part in the high rate at which urine is formed, the rate of tubular water and chloride reabsorption being partly compensated by the enhanced rate at which water and chloride are filtered.

## DISCUSSION

A common feature in all the experiments with diuretics reported was the increase in the rate of glomerular filtration. But, while in some series this increase was not related to the urine flow, in others it was significantly correlated with the urine flow.

An increase in glomerular filtration rate of constant magnitude at all levels of urine flow was found in rats injected with a non-diuretic dose of mersalyl ( $0.0006$  mM/100 g) and in rats which received 50 ml/100 g of an 85 mM NaCl solution. There was no increased diuresis in these series: the "mersalyl rats" excreted about 80% of the standard amount of water in 3 hours, and the "NaCl animals" only 63%. However, the mean values for GFR in both series were comparable, the difference in the volume of urine excreted during the period of 3 hours must therefore have been due to the difference in the rate at which water was reabsorbed. It can therefore be concluded that, with a glomerular filtration rate not correlated with the urine flow, the regulation of the urinary volume will depend on variations of the rate of water reabsorption, even when GFR is nearly three times as high as in normal rats.

This finding should be compared with those experiments in which the rate of glomerular filtration was correlated with the urine flow, i.e., in rats injected with

0.0027 mM /100 g mersalyl, with 0.01 mM /100 g theophylline sodium acetate, or with 0.00027 mM /100 g calomel. In these rats the rates of glomerular filtration were practically the same at the same rate of urine flow, and statistical analysis showed that the correlation coefficients between GFR and rate of urine flow of the three series were not significantly different and were, therefore, comparable. It could further be shown that variations in urine flow in these series were the result of variations in two functions: variations in the glomerular filtration rate (GFR) and variation in tubular water reabsorption ( $T_w$ ), instead of that in one ( $T_w$ ) only, as in the rats with a constant GFR. It may be asked: do variations in the glomerular filtration rate necessarily have a diuretic effect? It has been shown that calomel was not diuretic, but that both theophylline and mersalyl had a diuretic effect. However, Table I shows that the values for the glomerular filtration rates were much the same in all three series. It follows that the diuretic effect of theophylline and of 0.0027 mM /100 g mersalyl and the absence of a diuretic effect of calomel must have been produced by changes in the rate of water reabsorption ( $T_w$ ). If this is so, it should be possible to show that less water had been reabsorbed in rats injected with 0.0027 mM /100 g mersalyl than in rats which received theophylline, and that more water had been reabsorbed in rats injected with calomel. That this occurs may be seen from a comparison of the three coefficients of correlation between rate of water reabsorption and rate of urine flow (for the mersalyl series,  $r = -0.97$ , for the theophylline series,  $r = -0.68$ , and for the calomel series,  $r = -0.54$ ). It can thus be concluded that in rats injected with calomel no diuretic effect is exerted because the rate of tubular water reabsorption is increased to such an extent that it counterbalances the correlated increase in the rate of filtration. On the other hand, in rats injected with 0.0027 mM /100 g mersalyl or with theophylline, the rate of tubular water reabsorption increased to a lesser degree, and insufficiently to counterbalance the increased rate at which water was filtered.

It follows that the classical explanation that the diuretics used produce an enhanced diuresis by reducing the tubular water reabsorption applies also to the rat—provided that the rate of tubular water reabsorption obtained in experiments on rats is not compared with the rate of tubular water reabsorption of the controls. All the drugs investigated produced an increase in the rate of tubular water reabsorption, as expressed in percentage of glomerular filtration rate, if compared with that observed in a normal water diuresis in control rats. However, it would clearly be a mistake to compare a diuresis experiment in which only one variable ( $T_w$ ) is involved with one in which two variables ( $T_w$  and GFR) operate, i.e., experiments in which an increase of urine flow is determined by two different mechanisms.

Considering the increases in GFR and RPF, it remains to be seen how far the increase in chloride excretion observed in some of the series should be related

with the rate of tubular chloride reabsorption ( $T_{Cl}$ ). A significant increase of  $T_{Cl}$  was noted after a diuretic dose of mersalyl and after the injection of calomel and theophylline. Theophylline produced a greater chloride excretion than calomel, but the highest chloride concentrations were met after the injection of 0.0027 mM/100 g mersalyl. Two factors were involved in the increased excretion of chloride: (a) an increase in the plasma chloride concentration which occurred in rats injected with calomel or with a diuretic dose of mersalyl, (b) the rate at which the chlorides were reabsorbed. The rates of filtration were much the same in the three series, and it is therefore easy to understand why theophylline, which did not produce an increase of the plasma chloride level, produced a lower chloride excretion than 0.0027 mM/100 g mersalyl. After the injection of the higher dose of mersalyl or of calomel, plasma chlorides and rate of glomerular filtration were raised to approximately the same extent, and the amounts of chloride filtered were therefore comparable. It could thus be expected—and this was actually found in the calomel experiments—that a higher rate of tubular chloride reabsorption would result in a lower concentration of chloride in the urine.

It might be objected that expressing tubular water and chloride reabsorption as percentage of water and chloride filtered ( $T_w$  and  $T_{Cl}$ ) is a misleading way of describing these renal functions, and that absolute amounts of chlorides or water should be given in preference (Barclay and Cooke, 1944). But it would be an obvious mistake to divorce the quantity of chloride reabsorbed from that which is made available by filtration, and to deny the fact expressed by Shannon (1942) that "the burden presented to the tubules is quantitatively determined by composition and rate of formation of glomerular filtrate." Expressing the rate of water and chloride reabsorption as absolute amounts may be a permissible simplification when the GFR remains constant. However, it has been shown (Chasis, Ranges, Goldring, and Smith, 1938; Shannon, 1936) that this does not always apply even in man or the dog.

How do the results on rats compare with those obtained on isolated kidneys, or on dogs or man? Schmitz (1932), Blumgart, Gilligan, Levy, Brown, and Volk (1934), Davenport, Fulton, Van Auker, and Parsons (1934), Walker, Schmidt, Elsom, and Johnston (1937) found by means of creatinine and urea clearance determinations in anaesthetized animals and in man, that salyrgan, a proprietary brand of mersalyl, did not increase the glomerular filtration rate, and that the renal plasma flow, as measured with a thermostromuhr, was not affected in a constant manner. In contrast to this, it has been shown that a diuretic dose of mersalyl in rats produces an increase in both glomerular filtration rate and renal plasma flow when estimated by simultaneous inulin and diiodone clearances. From the findings in man and in the dog it was furthermore concluded that the mercurials act on the kidney by reducing the tubular water reabsorption. It could be shown that this also applies to rats, provided that the rate of water reabsorption in

rats injected with a non-diuretic dose of a mercurial "diuretic" is chosen as basis of comparison, and not that of control animals. It would seem, therefore, that, while in higher mammals the diuretic action of mersalyl is achieved by changes in one renal function only, i.e., in tubular water reabsorption, the diuretic action in the rat is more involved and is produced by changes in both the rate of glomerular filtration and the rate of water reabsorption.

It has been demonstrated that these renal effects of mersalyl on rats are the result of the mercurial constituent only, and not of the theophylline content of this preparation. Renal effects of mersalyl have been observed 10 hours after the injection, i.e., at a time when any diuretic action of theophylline had long subsided.

No indication of a central action of theophylline at the doses given was observed, and its renal effects were much the same as those observed by Verney and Winton (1930) in the heart-lung-kidney preparation, and those found by Smith and collaborators (1938) in man. These authors, who used inulin clearances, observed that theophylline produced an increase of the glomerular filtration rate which was correlated with the urine flow. Another analogy with their findings consists in the absence of a diuresis without previous hydration. It can therefore be concluded that the mechanism of the diuretic effect of theophylline sodium acetate is comparable in species as widely different as man, the dog, and the rat, but that a mercurial compound like mersalyl produces a diuretic action in rats by the combination of changes in the rate of glomerular filtration and of tubular water reabsorption.

#### SUMMARY

1 The injection of 0.0006 mM /100 g. mersalyl had no diuretic effect in rats, but it increased the glomerular filtration rate.

2 A marked diuretic effect was obtained with 0.0027 mM /100 g. mersalyl. This diuretic effect was obtained about 10 hours after the intramuscular injection of the mercurial compound and only in well-hydrated rats.

3 Ten hours after the administration of 0.0027 mM./100 g. mersalyl the glomerular filtration rate and the renal plasma flow were increased and significantly correlated with the urine flow.

4 Injections of 0.00027 mM /100 g., 0.00135 mM./100 g., and of 0.0027 mM /100 g. calomel failed to exert any diuretic effect. Rats injected with the two latter doses remained almost completely anuric for several hours.

5 The injection of 0.00027 mM /100 g. calomel produced an increase of glomerular filtration rate which was significantly correlated with the urine flow. The renal plasma flow was also increased, but not correlated with the urine flow.



6 The injection of a dose of 0.01 mM / 100 g theophylline sodium acetate in well-hydrated rats exerted a marked diuretic effect. Both glomerular filtration rate and renal plasma flow were increased and significantly correlated with the urine flow.

7 After injection of (a) a non-diuretic dose of 0.0006 mM / 100 g mersalyl, (b) a diuretic dose of 0.0027 mM / 100 g mersalyl, (c) a non-diuretic dose of 0.00027 mM / 100 g calomel, and (d) a diuretic dose of 0.01 mM / 100 g theophylline sodium acetate, the tubular water reabsorption (expressed as percentage of the glomerular filtration =  $T_w$ ) was increased when compared with that of control rats. However, a comparison of the rate of tubular water reabsorption in rats injected with a diuretic dose of 0.0027 mM / 100 g mersalyl or of 0.01 mM / 100 g theophylline, with that in rats injected with a non-diuretic dose of a mercurial compound, showed that the diuretic effect was accompanied by a reduction in the rate of tubular water reabsorption. It would seem, therefore, that in rats the diuretic effect of mersalyl and of theophylline sodium acetate was produced by changes in the rate of both the glomerular filtration and tubular water reabsorption.

I should like to express my sincere thanks to Dr H Heller for his stimulating criticism.

I am indebted to the Medical Research Council for a personal grant. The expenses of the work were defrayed by a Colston Research Grant.

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# METHOD FOR THE ESTIMATION OF BARBITURIC AND THIOBARBITURIC ACIDS IN BIOLOGICAL MATERIALS

BY

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In preliminary work on the fate and distribution of barbiturates in the animal body several known methods of determining them were tried (Levy, 1940, Delmonico, 1939, Anderson and Essex, 1943), but none was completely satisfactory. With these methods the recoveries of known amounts of barbiturates added to samples of blood and tissues were low.

A method has been developed for the estimation of barbituric acids based on Koppanyi's colour reaction (1934) and for the estimation of thiobarbituric acids based on Cowan's colour reaction (1939). The main feature of this method is the purification of the extracts containing barbituric and thiobarbituric acids by chromatography, which has the additional advantage of permitting the determination of both types of compounds when present together in the same sample.

The method involves the following stages: (i) extraction of the drugs from the biological material; (ii) purification of the extracts by chromatography; and (iii) determination of the drug content in the eluates.

## REAGENTS

- 1 Peroxide-free ether. Ether (technical) was treated overnight with ferrous sulphate, washed with water, dried with anhydrous calcium chloride and distilled over sodium.
- 2 10 per cent (w/v) solution of sodium dihydrogen phosphate.
- 3 Crystalline sodium dihydrogen phosphate.
- 4 Anhydrous sodium sulphate.
- 5 Chloroform free from alcohol. Chloroform (B.P.) was washed with water and then with a saturated solution of calcium chloride. After drying with anhydrous calcium chloride it was distilled and kept in a dark bottle.

6 Methanol A R quality

7 Benzene A R quality

8 Activated alumina 1,200 g activated alumina, 'grade O,' supplied by Messrs Peter Spence, Manchester, were boiled for 2 hours with 1 800 ml 10 per cent (v/v) acetic acid. The alumina was filtered and the excess of acetic acid removed by washing with at least 20 l of hot distilled water. The alumina was dried and reactivated by heating until the temperature reached 360° C, it was then partly deactivated by adding water (2.5 per cent w/v).

9 For the estimation of barbituric acids (a) 1 per cent (w/v) cobalt acetate in methanol and (b) 5 per cent (v/v) isopropylamine in methanol

10 For the estimation of thiobarbituric acids (a) a saturated solution of anhydrous copper sulphate in methanol and (b) 10 per cent (v/v) diethylamine in methanol

### PROCEDURE

**EXTRACTION—Blood**—10–20 ml volumes of oxalated blood are mixed with equal volumes of water and of the sodium dihydrogen phosphate solution and extracted with ether in a continuous extractor (at 45–50° C) for 8–10 hours. The ether extract is evaporated to dryness.

**Urine**—The total or an aliquot of the urine is acidified with conc HCl to pH 5. It is then extracted with ether in a continuous extractor (at 45–50° C) for 8–10 hours and the extract evaporated to dryness.

**Tissues**—Ether extracts of tissues are difficult to purify by chromatography, but the following method of extraction has been used with success.

Samples of about 10–20 g of tissues are ground in a mortar with sand and then mixed with solid sodium dihydrogen phosphate (1 g for every 10 g of tissue) and allowed to stand for 5–10 minutes. Anhydrous sodium sulphate (20 g for every 10 g of tissue) is then added slowly, with continuous grinding, to give a fine homogeneous powder. The whole is transferred to a desiccator and left over anhydrous calcium chloride for one hour. The dry powder is extracted for 2–3 hours with 50 ml benzene in a well-stoppered 100 ml conical flask. The benzene extract is then filtered and the residue and flask washed three times with about 10–15 ml of benzene. The filtrate and washings are pooled and concentrated to about 5 ml in a distillation flask at 50° C under reduced pressure. This method of extraction can also be applied to blood.

### PURIFICATION OF THE EXTRACTS AND SEPARATION OF BARBITURIC FROM THIOBARBITURIC ACIDS

This is based on the work of Kondo (1937), who separated barbitone from phenazone by chromatography on alumina columns.

**Urine and blood**—The residues left by evaporation of the ether extracts are dissolved in 5 ml chloroform and dried by shaking with about 1–2 g anhydrous sodium sulphate. The solutions are then chromatographed on alumina columns (3/8" × 4"). The chloroform solutions are filtered directly on to the columns, the

flask and filter are washed three times with 5 ml of chloroform and the washings poured on to the column. The column is then washed with chloroform until the eluates are free from pigment.

*Tissues*—The benzene tissue extracts are passed through alumina columns, with slight suction. The flasks are washed three times with benzene and the washings added to the column. The column is then washed with benzene until the eluates are free from pigment and finally with 20 ml chloroform.

The chloroform and benzene eluates are discarded. If the extracts contain more than 2 mg of a thiobarbituric acid, the latter can be seen under ultraviolet light as a dark band at the top of the column.

*Separation*—Thiobarbituric acids are recovered from the columns by elution with 50 ml 2 per cent methanol in chloroform (v/v). Barbituric acids are not eluted by methanol and chloroform in this proportion but they can be recovered by further elution with 50 ml 10 per cent methanol in chloroform (v/v). These eluates are kept for estimation.

The separation of thiobarbituric acids from barbituric acids is complete and the recoveries of both fractions are almost theoretical. Mixtures containing 0.25–0.5 mg thiophenobarbitone and 0.25–1 mg phenobarbitone were added to alumina columns, and the average recoveries were thiobarbituric acid 102 per cent and barbituric acid 98 per cent.

*ESTIMATION*—The eluates from the columns are evaporated to dryness in distillation flasks under reduced pressure at 40–50° C. The residues are dissolved in chloroform and their barbituric or thiobarbituric acid content estimated by the following reactions.

*Thiobarbituric acids*—Thiobarbituric acids are estimated by a modification of the reaction demonstrated by S. L. Cowan at the Physiological Society in 1939. An aliquot of the final chloroform solution is taken in a test tube and for every 2 ml, 0.2 ml of the diethylamine solution and 0.5 ml of the copper sulphate solution are added in that order. A green coloration develops at once which is stable for about two hours. The samples are compared in a colorimeter, photoelectric or otherwise, with a series of similarly treated standard solutions of the thiobarbituric acid to be estimated, containing from 0.03–0.5 mg/ml. These are prepared by diluting a fresh solution containing 0.5 mg/ml of the thiobarbituric acid in chloroform.

Extracts of tissues, such as brain and liver, give a slight blank with the copper reaction for thiobarbituric acids. This can be as high as 1 mg/100 g of tissue and it is necessary to subtract this blank from the estimations.

The reaction is fairly specific. According to Cowan (personal communication) it is not given by malonic acid, theophylline, theobromine, thiourea, caffeine, guanine, uric acid, urea, creatinine, oxamide, succinic acid, lecithin, cholesterol, cystine or glutathione.

Barbituric acids give a faint bluish colour under the conditions described above. The intensities of the colours given by standard solutions of phenobarbitone and thiophenobarbitone are compared in Fig 1

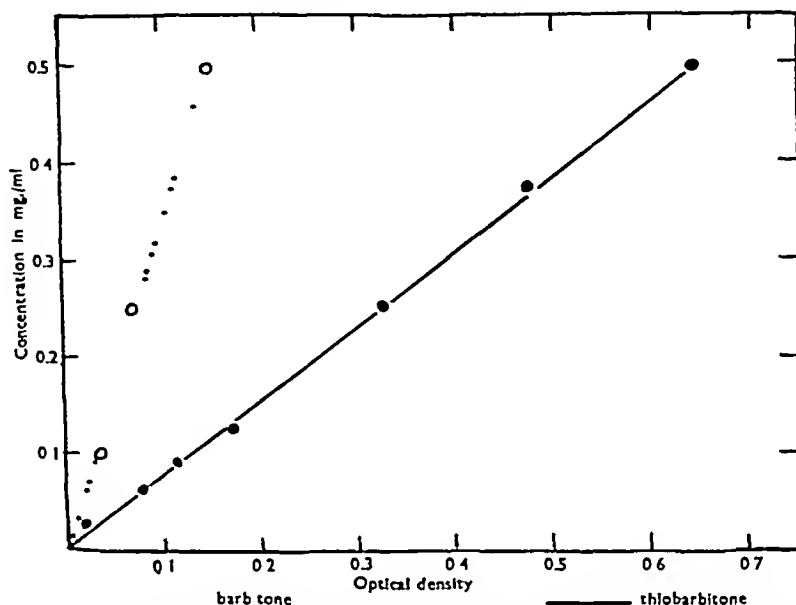


Fig. 1—Intensity of the colour given by solutions of barbitone and thiobarbitone in Cowan's reaction. Measurements made in the Pulfrich photometer with filter 66, 6/3.5 and 10 mm cells

The Pulfrich photometer was used in these estimations. With filter S 66, 6/3.5, maximum transmission at 679 mμ and 1 cm cells, the reaction is sensitive to concentrations of 0.03 mg/ml, but the sensitivity can be increased to 0.005 mg/ml if 5 cm cells are used.

**Barbituric acids**—These are estimated by Koppanyi's reaction (1934). For every 2 ml of the final chloroform solution, 0.6 ml isopropylamine and 0.1 ml cobalt acetate reagents are added. The reddish colour given by the sample is compared in a colorimeter with a series of similarly treated standards containing 0.1–1.0 mg of the appropriate barbituric acid per ml chloroform.

**Recovery**—The method was tested in a series of control experiments in which known amounts of sodium kemithal (5-Δ<sup>2</sup>-cyclohexenyl-5-allyl-2-thiobarbituric acid) were added to samples of blood and tissue and treated as described above. The results of these experiments are summarized in Table I.

The recovery of known amounts of barbituric or thiobarbituric acids added to samples of blood and tissues is approximately complete except when the amount in the 10 ml sample is less than 0.3 mg., when the recovery may fall below 95 per cent.

TABLE I

RECOVERIES OF 5- $\Delta^{1,2}$ -cycloHEXENYL-5-ALLYL-THIOBARBITURIC ACID (KEMITHAL) FROM BLOOD AND TISSUES

Tissue	Tissue in g Blood in ml	Na kemithal added mg	Equivalent to kemithal acid mg.	Kemithal acid found mg.	Per cent recovery
Blood	10	8.0	7.15	6.5	91
	10	8.0	7.15	6.9	96
	10	8.0	7.15	7.2	103
	10	1.125	1.0	0.98	98
	10	1.125	1.0	1.01	101
	10	1.0	0.89	0.86	97
	10	1.0	0.89	0.86	97
	10	0.5	0.445	0.43	96.5
	10	0.5	0.445	0.42	94.5
	10	0.5	0.445	0.45	101
Liver	10	0.884	0.788	0.69	89
	10	0.884	0.788	0.75	97
	10	1.768	1.576	1.42	91
	10	1.768	1.576	1.47	95
	10	1.125	1.0	0.96	96
	10	1.125	1.0	0.98	98
	10	1.125	1.0	0.97	97
	10	1.125	1.0	0.96	96
Brain	10	1.0	0.89	0.86	97
	10	1.0	0.89	0.91	102
	10	1.0	0.89	0.84	94.5
	10	1.0	0.89	0.94	105
	10	1.0	0.89	0.84	97
	10	2.0	1.78	1.70	95.5
	10	2.0	1.78	1.88	105
	10	2.0	1.78	1.80	101

Average recovery per cent  $97.3 \pm 4$ 

## SUMMARY

A method for the estimation of barbituric acids and thiobarbituric acids in tissues and animal fluids is described.

The method enables both types of barbiturates to be separated and estimated when they are present together.

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# KEMITHAL

## A NEW INTRAVENOUS ANAESTHETIC

BY

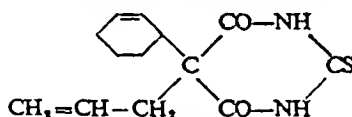
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(Received June 10 1946)

Since the introduction of evipan (now hexobarbitone B P) by Weese and Scharpff (1932) and pentothal by Lundy (1935), the use of the ultra-short-acting barbiturates for intravenous general anaesthesia has steadily increased. The literature on this subject has been reviewed by Adams (1944).

An extensive investigation on new thiobarbiturates has led to the discovery of a drug which has pharmacological advantages over those already in use. This



substance, to which the name of kemithal has been given, is 5- $\Delta^2$ -cyclohexenyl-5-allyl-2-thiobarbituric acid. The free acid, m.p.  $142^\circ \text{C}$ , is sparingly soluble in water but readily soluble in most organic solvents, e.g., alcohols, ether, chloroform, benzene, etc. Its sodium salt, a pale yellow slightly hygroscopic powder, is readily soluble in water up to 20 per cent. The pH of a 10 per cent (w/v) solution is 10.6 (cf. evipan, pH 11.5, pentothal, pH 10.6). The sodium salt is stable in absence of air; its solutions are stable for 4-5 hours.

This paper describes the pharmacological investigation of the drug. The results of its clinical trials on 4,000 cases have been reported elsewhere (Mackintosh and Scott, 1946; Halton, 1946; Gordon, 1946).

### *Hypnotic action and toxicity*

In this paper hypnosis is defined as that condition in which it was not possible to elicit "body righting reflexes" (Fulton, 1938). The term anaesthesia defines the state during which it is possible to make a cutaneous incision without evoking a response.

**Mice**—The median hypnotic dose (HD 50) and the median lethal dose (LD 50) of the three drugs were measured after intravenous, intraperitoneal, and oral administration. At least twelve mice were used at each dose level. For one hour before, and during the first



24 hours of the experiments, the mice were kept in thermostatically controlled cages at 30°C, they were then transferred to a room maintained at 25°C, and kept under observation for a week. The HD 50 and LD 50 were calculated from the incidence of hypnosis and death by the usual statistical methods. The term therapeutic ratio is used to denote the quotient LD 50/HD 50, and is an approximate indication of the safety margin of the drugs. The times of onset of hypnosis and duration of action were recorded. The drugs were administered as 0.1 per cent (w/v) or 1.0 per cent (w/v) solutions. Intravenous injections were carried out at a constant rate of 0.05 ml per 5 seconds. The results are summarized in Table I.

TABLE I  
HYPNOTIC ACTION AND TOXICITY IN MICE

Administration	Kemithal			Pentothal			Hexobarbitone		
	i v.	i p	oral	i v	i p	oral	i v	i p	oral
HD 50 (mg./kg.)	55	100	165	20	42	—	30	47	—
LD 50 (mg./kg.)	390	384	370	80	154	600	190	280	1200
Therapeutic ratio									
LD 50									
HD 50	7.1	3.84	2.25	4	3.65	—	6.3	6	—
Time of onset of hypnosis in min.	at once	3	2-5	at once	3	—	at once	4	—
Duration of action of 2 x HD 50 in min.	30-90	30-45	3hr	30-90	30-60	—	45-60	50-60	—

*Monkeys*—The duration of hypnosis produced by the intravenous administration of 5 per cent (w/v) or 10 per cent (w/v) solutions of the drugs in 3 monkeys (*M. rhesus*) was studied. In order to avoid habituation, the monkeys were only injected once a week, and the order of the injections of the different drugs was randomized. A range of doses from 5 mg./kg. to 100 mg./kg. was tried and Table II shows those which produce anaesthesia of 10 and 60 minutes' duration.

*Dogs*—The technique was similar to that used in monkeys. Hexobarbitone is not a satisfactory anaesthetic for dogs, and when anaesthesia was obtained it was associated with convulsions and delayed recovery. Thus in one dog 40 mg./kg. hexobarbitone produced hypnosis without loss of the corneal reflex. The recovery, which began 20 minutes after the injection, was only complete after 100 minutes, convulsions occurred throughout this period. A comparison of kemithal and pentothal is given in Table II.

TABLE II  
ANAESTHETIC ACTION IN MONKEYS AND DOGS

Animal		Kemithal	Pentothal	Hexobarbitone
Monkeys ( <i>M. rhesus</i> )	i v Dose (mg./kg.) producing 10 min. anaesthesia	22.5	11.5	20
	i v Dose (mg./kg.) producing 60 min anaesthesia	45	30	45
Dogs	i v Dose (mg./kg.) producing 10 min. anaesthesia	50	20	—
	i v Dose (mg./kg.) producing 60 min. anaesthesia	80	40	—

*Respiratory volume*

The changes in respiratory volume following kemithal and pentothal were measured in the unanaesthetized rabbit by Gaddum's method (1941), using a specially constructed rubber mask fitting closely to the head of the animal and connected through a set of valves to the recording apparatus. Solutions of the drugs were injected into the marginal vein of the ear, several doses being administered at intervals sufficiently long to allow the respiration to return to normal. The results are shown in Fig 1, which indicates that a dose of about 2.5 mg/kg

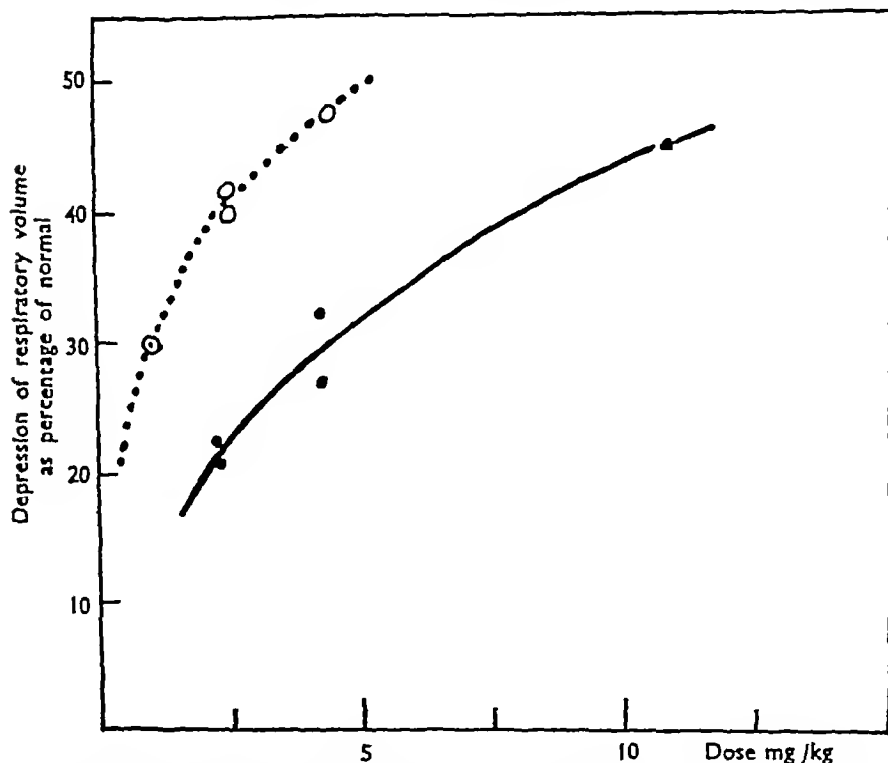


Fig 1—Depression of respiratory volume of the unanaesthetized rabbit following i.v. administration of kemithal and pentothal. Line—kemithal Dots—pentothal.

pentothal produces a depression of respiratory volume equivalent to that produced by 8 mg/kg kemithal. Experiments on decerebrate cats gave similar results.

*Blood pressure*

The effects of kemithal and pentothal on the blood pressure of cats, anaesthetized with chloralose or after decerebration, were compared. Solutions of both compounds (10 per cent w/v) were injected into the femoral vein, and the

blood pressure was recorded from the carotid artery, the animals being kept under artificial respiration throughout the experiments. Both compounds produced a fall of blood pressure, 10 mg/kg kemithal produced approximately the same fall of blood pressure as 5 mg/kg pentothal.

#### *Rate of inactivation of kemithal in mice*

The rate of inactivation of kemithal in mice was measured by the continuous intravenous injection method described by Das and Raventós (1939). The mice received an initial intravenous dose of 80 mg/kg kemithal followed by a continuous injection in a series of doses, calculated in mg/kg/min and adjusted by alteration of the concentration of the solution, the rate of the injection being kept constant at 0.02 ml/min. The rate of inactivation was calculated from the dose in mg/kg/min. necessary to maintain the level of anaesthesia produced by the initial injection for a period of 90–120 minutes. The results are summarized in Table III.

TABLE III  
RATE OF INACTIVATION OF KEMITHAL IN MICE

Dose mg./kg./min.	Number of mice	Average time of recovery in minutes	Average time of death in minutes	Remarks
0.8	3	5	—	Progressive decrease in depth of anaesthesia
1.5	3	12	—	Ditto
2.0	5	90	—	Slight decrease in depth of anaesthesia followed by increase 40–50 min. after the beginning of the continuous injection. This level of anaesthesia is maintained for more than 90 min.
4.0	3	—	45	Progressive increase in depth of anaesthesia followed by death
5.0	2	—	40	
7.5	3	—	35	
15.0	3	—	22	
20.0	3	—	14	

From these results the rate of inactivation is calculated as follows:

Initial dose = 80 mg/kg

Dose for maintenance of anaesthesia = 2 mg/kg/min

Rate of inactivation per minute =  $\frac{2}{80}$ , i.e., 2.5 per cent of the initial dose

#### *Blood concentration of kemithal during anaesthesia*

The concentration of kemithal in the blood during anaesthesia was studied in rabbits and men. The drug concentration was measured by the method described in the previous paper (Raventós, 1946), which can be applied to any barbituric acid derivative.

Rabbits of 2-3 kg received large doses of kemithal (100-130 mg/kg) by slow intravenous injection over a period of 3 to 5 minutes. In some cases temporary arrest of the respiratory movements occurred and the animals were kept under artificial respiration until spontaneous respiratory movements were re-established. Samples of blood were taken from the femoral artery at intervals.

Immediately after administration, the blood concentration of kemithal was 15-18 mg/100 ml, falling to 5-7 mg/100 ml in 15 minutes. From this point the blood concentration decreased progressively for a further 75 minutes until it reached levels of 2-2.5 mg/100 ml. After this fall of concentration, the disappearance of the drug from the blood became slower and 1.25-1.75 mg/100 ml of kemithal were found in the blood 3-4 hours after injection. A comparison of the results in one experiment with kemithal with those obtained in one experiment with hexobarbitone is shown in Fig 2. In rabbits, recovery begins when the blood concentration of kemithal is 2.0-1.5 mg/100 ml.

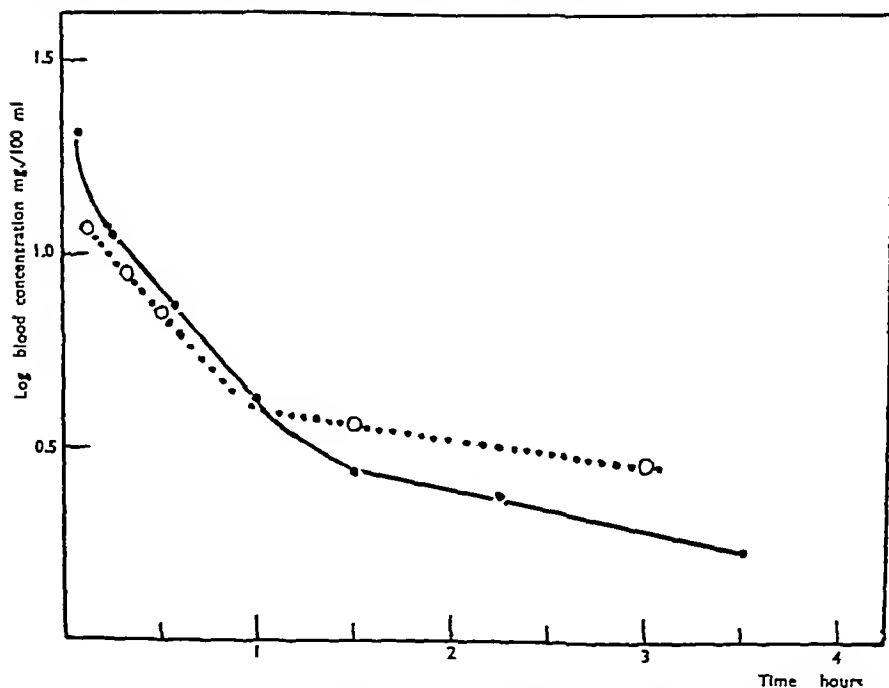


Fig 2.—Blood concentration of kemithal and hexobarbitone in the rabbit during anaesthesia. Line—kemithal Dose, 130 mg./kg i.v. Dots—hexobarbitone Dose, 130 mg./kg. i.v.

Changes of the concentration of kemithal in the blood of men under anaesthesia were studied in patients under the care of Dr J Halton, Liverpool. An initial dose of 1.5 or 2.0 g kemithal was injected intravenously and anaesthesia was maintained with cyclopropane. Blood samples of at least 20 ml were taken at intervals from the cubital vein.

When 1.5 g kemithal were administered, the initial blood concentration was 2.3–3.5 mg/100 ml. Immediately after the administration of 2.0 g. kemithal the blood concentration was 3.5–4.5 mg/100 ml and it decreased progressively to 1.2–1.9 mg/100 ml in about 45 minutes. It was not possible to follow the changes in blood concentration after the end of the operation. The fall in blood concentration during the first 45 minutes of one of these experiments is shown in Fig. 3.

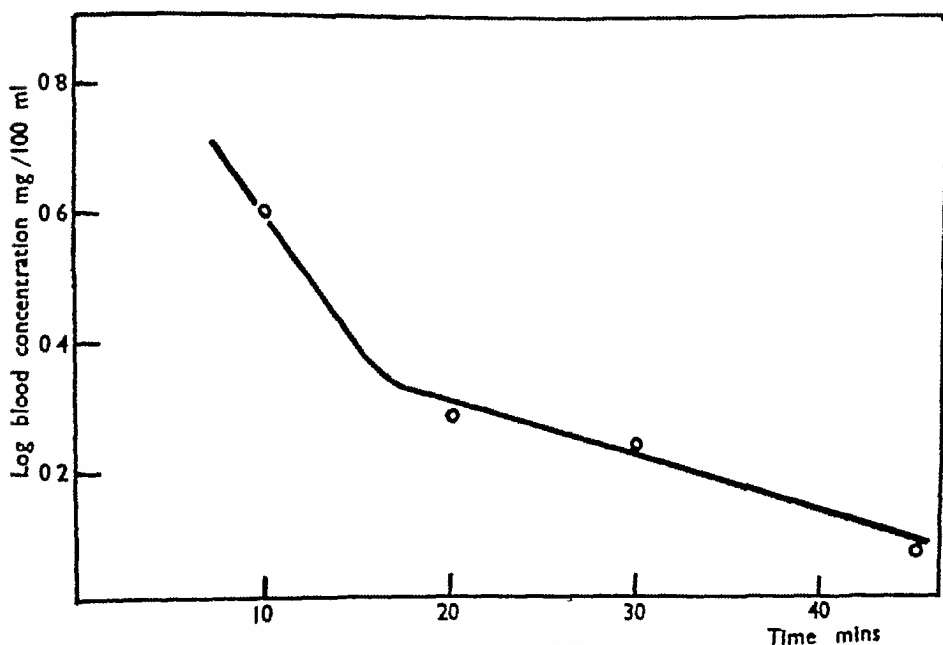


FIG. 3—Blood concentration of kemithal in a 60-kg man after a dose of 2.0 g. Anaesthesia maintained with cyclopropane.

#### *Relationship between the blood concentration of kemithal and the level of anaesthesia*

The depth of anaesthesia produced in rabbits by a continuous intravenous injection of 3 mg/kg/min. kemithal, a dose large enough to produce an accumulation of the drug, was compared with the blood concentration. The solution was injected at a rate of 0.15 ml/min. Samples of blood were taken from the femoral artery as the anaesthesia deepened, at the levels of light and surgical anaesthesia and at the arrest of respiratory movements. Light anaesthesia is assessed as a level of anaesthesia sufficient for a painless incision of the skin, surgical anaesthesia is a level of anaesthesia sufficient for the opening of the peritoneal cavity with good abdominal relaxation. Similar experiments were carried out with pentothal, injected continuously at a dose of 2 mg/kg/min.

The average blood concentrations of kemithal and pentothal at these levels of anaesthesia are shown in Table IV. In the same table the ratios between these concentrations give a further indication of the safety margin of the compounds.

TABLE IV

THE RELATIONSHIP BETWEEN BLOOD CONCENTRATION AND LEVEL OF ANAESTHESIA

Drug	Number of experiments	Average drug concentration in the blood in mg./100 ml			Ratios		
		Light anaesthesia A	Full surgical anaesthesia B	Respiratory arrest C	$\frac{C}{A}$	$\frac{C}{B}$	$\frac{B}{A}$
Kemithal	8	3.55	8.82	18.8	5.28	2.13	2.44
Pentothal	7	2.7	4.78	7.42	2.74	1.55	1.77

### Excretion

A total of 750 mg kemithal, divided into three equal doses administered at two-hourly intervals, was injected intravenously into 2-3 kg. rabbits and the urine collected during the three following days. The excretion of thiobarbituric and barbituric acids was estimated for each of the three days.

In these experiments about 15 mg kemithal were recovered in three days, accounting for only 2 per cent of the administered material. In addition to the recovery of this small amount of unchanged thiobarbiturate, substances giving a positive reaction in the barbituric acid test were excreted to a total of about 20 mg, equivalent to 2.5 per cent of the dose. Nearly all this material was excreted during the first 24 hours following injection, as shown in Table V.

TABLE V

EXCRETION OF KEMITHAL BY RABBITS AFTER  $3 \times 250$  MG I.V.

Day	Urinary excretion of		Total
	Thiobarbituric acid (kemithal) mg	Barbituric acid, as 5- $\Delta^{21}$ -cyclohexenyl-5-allyl-barbituric acid mg.	
1	14.87	16.50	31.37
2	1.17	3.05	4.22
3	0.13	0.79	0.92
Total	16.17	20.34	36.51
Per cent excreted	2.15	2.7	4.85

Man also excretes only a very small proportion of unchanged kemithal. In one of Dr Halton's cases, receiving 60 g kemithal by drip, only 46 mg of thiobarbituric acid were recovered from the urine during the first 24 hours after anaesthesia.

### Histology

Microscopical sections of tissues of rabbits injected with 50 mg kemithal daily for two weeks have been examined. At the end of this period the animals were killed and the specimens fixed in formol-saline, embedded in paraffin, and the sections stained with haematoxylin and eosin. The liver, kidney, spleen, lung, intestine, pancreas, suprarenal and heart of these animals showed no pathological changes.

### DISCUSSION

As an intravenous anaesthetic in mice, monkeys and dogs, kemithal is about one-half as active, weight for weight, as pentothal, and slightly less active than hexobarbitone. The therapeutic ratio in mice is, however, markedly greater than that of either of the other drugs. The times of onset and maintenance of anaesthesia and the rates of recovery are similar in equiactive doses in all the animals tested, except that hexobarbitone gives anomalous and unsatisfactory results in dogs. The therapeutic ratio of kemithal in mice varies considerably with the method of administration: as shown in Table I, the LD 50 remains approximately the same irrespective of the route of administration, but the HD 50 i.p. is approximately twice, and the HD 50 oral three times, the HD 50 i.v., whereas with pentothal and hexobarbitone the changes in HD 50 and LD 50 vary *pari passu*.

The favourable intravenous therapeutic ratio of kemithal was confirmed in the experiments on the blood concentration in rabbits at different levels of anaesthesia: it was shown that the ratios of the concentrations at the arrest of respiratory movements to those at both full surgical and light anaesthesia are considerably greater with kemithal than with pentothal (Table IV). Thus kemithal appears to have a greater factor of safety than pentothal whatever the level of anaesthesia. It is difficult to make a precise correlation between the levels of anaesthesia used in the rabbit and the stages of anaesthesia in man as laid down by Guedel (1937), but the level of "light anaesthesia" would correspond approximately to Guedel's first plane of the third stage and "full surgical anaesthesia" to his third plane of the third stage.

The depression of the respiratory volume produced by kemithal, both in rabbits and decerebrate cats, weight for weight, is one-third to one-quarter that of pentothal. As the equiactive dose of kemithal is only about twice that of pentothal, the advantage of kemithal in this respect is clear. The depressant action of kemithal on the blood pressure is similar to that of equiactive doses of pentothal.

In determining the rate of inactivation of kemithal in mice by continuous injection, it was found that about 2 mg /kg /min was required to maintain the level of anaesthesia induced by 80 mg./kg, i.e., about 25 per cent of the initial dose. This rate of inactivation is similar to that obtained by Das and Raventós (1939) for hexobarbitone in mice.

The disappearance of kemithal from the blood of rabbits which have received large intravenous doses falls into three main phases. During the first 10–15 minutes there is a rapid fall in blood concentration, which is clearly associated with the distribution of the drug throughout the body. During the second phase, up to 90 minutes after the injection, the concentration decreases progressively, falling to about one-half of the original concentration in 30–40 minutes, or at a rate of 2–2.5 per cent per minute. This figure is in close agreement with that of the rate of inactivation of kemithal found in mice by the continuous injection method. In the third phase, the fall in blood concentration is very much slower. The cause of this change is not clear, it may be due to the continuous slow removal of the drug from some depot after the main excretion is complete, or it may be caused by the formation of a metabolic product which interferes with the normal process of destruction of the drug. After the administration of hexobarbitone the same changes in rate of disappearance of drug from the blood were found.

In the experiments on the rate of inactivation of kemithal in mice injected with 2 mg /kg /min (Table III) it was observed that the level of anaesthesia tended to decrease during the first 30–45 minutes of the continuous injection, but later this level increased slightly and remained constant until the end of the experiments. We think this result is perhaps further evidence of the formation of an inhibitory metabolite.

In man the rate of disappearance of kemithal from the blood is about the same as in the rabbit. Kemithal is almost completely destroyed in the body. In rabbits only about 2 per cent of the dose administered can be detected in the urine, in man less than 1 per cent. In addition, the urine of rabbits after administration of kemithal contains substances which give Koppanyi's (1939) test for barbiturates, but not the test for thiobarbiturates, in quantities corresponding to 2–3 per cent of the administered kemithal. This may indicate that the inactivation of kemithal involves the removal of sulphur from the molecule.

#### SUMMARY

1 As an intravenous anaesthetic in mice kemithal (5- $\Delta^2$ -cyclohexenyl-5-allyl-2-thiobarbituric acid) is about half as potent as pentothal and slightly less active than hexobarbitone. It has a higher therapeutic ratio (LD 50/HD 50) than either of the other two compounds.

2 The duration of action of equiactive anaesthetic doses of the three compounds is about the same.



3 Kemithal in anaesthetic doses depresses the respiratory volume to a less extent than pentothal

4 The concentrations of kemithal and pentothal in the blood at different levels of anaesthesia and the ratios between these concentrations have been determined. They suggest that with kemithal anaesthesia can be obtained with less danger of respiratory arrest.

5 The rate at which kemithal and hexobarbitone disappear from the blood after administration has been determined in rabbits

6 Kemithal is almost completely destroyed in the body. The urine of rabbits injected with kemithal contains small amounts of the unchanged material and similar quantities of barbituric acids, leaving about 95 per cent of the administered dose to be accounted for

7 No histological changes have been found in the tissues of rabbits injected intravenously with 50 mg kemithal daily for two weeks

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# SOME PHARMACOLOGICAL AND CHEMOTHERAPEUTIC PROPERTIES OF NOTATIN

BY

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This paper includes descriptions of work carried out by colleagues in other member firms of the Therapeutic Research Corporation, such work is referred to under the name of the worker responsible. Fuller reference to these colleagues is made in the acknowledgment at the end of the paper.

The nature and some of the properties of notatin have been described briefly by Coulthard *et al* (1942) and Birkinshaw and Raistrick (1943) and in detail by Coulthard *et al* (1945). The substances "penatin" (Kochalaty, 1942, 1943) and "penicillin B" (Van Bruggen *et al*, 1943) are similar to, and probably identical with, notatin (Birkinshaw and Raistrick, 1943). Notatin is a flavoprotein enzyme catalysing the oxidation of glucose to gluconic acid by means of atmospheric oxygen, with the production of hydrogen peroxide. It exhibits high antibacterial activity *in vitro* in the presence of glucose, but is inactive in the absence of glucose or in the presence of catalase, the antibacterial activity therefore appears to be due to hydrogen peroxide formed during the oxidation of glucose.

The present paper is an account of some of the pharmacological properties of notatin, and of unsuccessful attempts to demonstrate an antibacterial action *in vivo*.

## *Acute toxicity and symptoms*

The acute toxicity of the purest samples of notatin yet obtained is indicated by the following approximate median lethal doses

		Mg per kg body weight
<i>Mice</i>	Intraperitoneal	3
	Subcutaneous	4.5
	Intravenous	13
<i>Rabbits</i>	Subcutaneous	7.5

The oral toxicity is low, doses up to 300 mg per kg have no effect.

The effect of fatal intravenous doses has been observed in dogs, cats, rabbits, rats, and mice. In a cat or dog an intravenous dose of about 30 mg. per kg rapidly produces severe cyanosis and anoxaemia. The haemoglobin of the blood is converted into a brown pigment which appears to be methaemoglobin, at the same time the blood becomes more viscous and the clotting time is reduced. Death occurs in a matter of minutes, and cannot be averted by artificial respiration. At autopsy the lungs are grossly oedematous, and in many cases the liver shows marked degeneration. The general picture is similar in rabbits, rats, and mice, with minor differences. On the other hand, intravenous doses which are not immediately fatal appear to have no effect whatever, this has been observed in cats, rabbits, and rats, with doses so large as to be only just sub lethal.

The effect of fatal subcutaneous doses is different from that of intravenous doses, a longer time elapses before death occurs (6 to 24 hours) and there is no observable blood-pigment change. The outstanding symptom in rabbits is a very marked rise in blood sugar, which is described below.

During the early stages of the production of notatin, as the samples obtained became successively purer, it was found that the toxicity became greater as the antibacterial activity *in vitro* became greater, indicating that the high toxicity of notatin is an inherent property of the active material and is not due to a separable impurity. This is confirmed by an observation by Miss Chapman that at least 90% of the toxicity disappeared when the antibacterial activity was destroyed by mild hydrolysis, the stimulant action on smooth muscle and the antidiuretic action in rats were similarly decreased.

#### *Effect on blood pigment*

The effect of large intravenous doses on the blood pigment was investigated in the rat and the rabbit, and considerable differences were observed between the two species. In the rat, severe cyanosis was evident immediately the dose was injected, and the blood samples taken at any time after the injection showed the characteristic absorption spectrum of methaemoglobin. In the rabbit, although the blood gradually became darker after injection and was very considerably darker at the time of death, no methaemoglobin was detectable except in animals which had been anaesthetized with ether. The pigment causing darkening of the blood in unanaesthetized rabbits was not identified.

The change to methaemoglobin could be produced in rabbit blood *in vitro*, provided glucose was added and heparin was used as the anticoagulant. Methaemoglobin was not produced in the absence of added glucose, and not in any case if oxalate was present. Lysed washed red cells were affected in the same way as whole blood.

The fact that the only rabbits in which methaemoglobin formation was observed *in vivo* were etherized animals may be linked with the finding that a high glucose concentration is necessary *in vitro*, since the etherization of a rabbit raises the blood sugar.

*Effect on carbohydrate metabolism*

The effect of notatin on the reducing substances in blood was investigated in rabbits, all estimations were done by the Hagedorn-Jensen method

After subcutaneous injection of a dose in the region of the median lethal dose, either the blood sugar rose gradually, sometimes reaching very high levels, and the animal eventually died, or else there was no observable effect whatever, the blood sugar remaining normal and the animal appearing to be quite unaffected in any way. The larger doses produced the former response, while the smaller doses were without effect, but it was observed more than once that a dose which caused a rise in blood sugar and subsequent death in one animal would have no effect in another animal. The borderline dose was about 7.5 mg/kg of the purest preparations

In those animals in which a blood sugar rise occurred, death usually took place from 20 to 30 hours after injection, the blood sugar having risen to a maximum level of 300 to 500 mg/100 ml., one or two animals died later than 30 hours. Only two animals survived after a rise of blood sugar, and in both cases the maximum blood sugar level reached was about 250 mg./100 ml., in no case did death occur without a blood sugar rise preceding it.

In several animals insulin was injected subcutaneously when the blood sugar was at its maximum, and the latter then fell rapidly. Unfortunately this phenomenon was difficult to observe, as the rabbit seemed unable to withstand the combined action of the two drugs, and invariably died soon after the injection of insulin. In a typical experiment the dose given was 7.5 mg./kg., and the blood sugar, initially 120 mg/100 ml., rose gradually until it was 300 mg/100 ml. 24 hours later at this point 16 units of insulin were injected subcutaneously, and the blood sugar fell rapidly, reaching 72 mg/100 ml. 5 hours later, 15 hours after this it had risen again to 150 mg/100 ml., and death occurred a little later.

Glycosuria was observed in some of the animals with high blood sugars, and the liver glycogen and body temperature fell as the blood sugar rose, as shown by the experiment recorded in Table I.

TABLE I  
EFFECT ON BLOOD SUGAR AND LIVER GLYCOGEN OF RABBITS

Treatment	Rabbit No	Blood sugar in mg./100 ml			Rectal temperature 9 hrs. after injection °F	Liver glycogen 9½ hrs after injection Per cent.
		Initial	4½ hrs after injection	9 hrs after injection		
Notatin 15 mg./kg. subcutaneously	1	115	273	455	94.0	0.26
	2	125	255	450	98.4	1.72
	3	126	204	368	97.8	1.36
Controls	4	136	125	191	—	3.86
	5	137	124	137	102.8	5.40
	6	119	103	112	101.6	4.80

After intravenous injection of notatin into unanaesthetized rabbits, a small fall in blood sugar usually occurred. No considerable rise was ever observed.

### Chronic toxicity

Rat growth tests carried out on five groups of five litter-mates of about 35 g weight showed that daily subcutaneous doses up to the largest tolerated dose (0.8 mg/kg), given for 14 days, had no effect on the growth rate.

A number of adult rabbits were given a daily subcutaneous dose of 2.5 mg/kg. for 14 days. There was no effect on the red cell count, haemoglobin or blood urea, but there was a marked granulocytosis which generally reached a maximum between the 5th and 8th days of dosage and then gradually returned to normal in spite of continuing daily doses. Rats given daily subcutaneous doses of 2 mg/kg for 20 days showed a similar granulocytosis.

The toxicity to leucocytes was examined by Mr Freeman, using a modification of the method of Thrower and Valentine (1943). Dilutions of notatin were prepared in normal citrated human blood and a staphylococcal suspension added. After incubation for 1 hour at 37°C, films were prepared and Gram-stained. The bacteria ingested into each of 25 phagocytes were counted, and a mean figure calculated. In the controls this was 21.4 cocci per phagocyte, and in notatin solution (1 in 200) 20.9 cocci per phagocyte. This concentration of notatin therefore did not inhibit phagocytosis.

Dr Ungar has observed that in tissue cultures of chick embryo heart, notatin added to the nutrient plasma slightly inhibited the growth of fibroblasts in dilutions from 1 in 10,000 to 1 in 1,000, but some growth still occurred at a dilution of 1 in 250.

### Local action

It was noticed throughout the experimental work that subcutaneous injections of notatin gave rise to oedematous swelling and pronounced tenderness at the site of injection. This was confirmed in *ad hoc* experiments on rabbits and rats, post-mortem examination showed widespread oedema, adhesions, and subcutaneous haemorrhage.

The action of notatin in uninfected wounds was observed in rats. The animals were anaesthetized and a small incision made in the skin of one leg; the underlying muscle was snipped with pointed scissors to a depth of 2 or 3 mm and the skin incision was ligatured, all under sterile conditions. The opposite leg was treated similarly, but notatin was introduced before ligation. In most animals, approximately 0.5 mg powdered notatin was introduced (about 2.5 mg per kg body weight) but in a few animals a solution was used (0.1 ml of 0.2 per cent, equivalent to 10 mg per kg. body weight). In three of the 12 rats used the foot distal to the notatin treated wound became grossly swollen and oedematous 4 to 6 days later, the use of and sensation in the limb being temporarily lost. The swelling began to subside in 10 to 12 days and the limb eventually returned to normal in 2 of the 3 animals (the third was killed for examination). Of these animals, two had been treated with notatin powder and one with a solution. In the remaining rats little or no swelling occurred and no difference in degree of healing was apparent between the treated and control wounds. No systemic effects were observed in any of the animals.

The action of notatin in an infected wound was observed in a single rabbit. Wounds were made in each leg as before and both were heavily, and as far as possible equally, infected with *Staphylococcus aureus* (Mrs Fox). One wound was then treated with 2 mg. powdered notatin. On the 4th day the notatin treated leg was oedematous. On the 7th day the treated wound was deeply necrosed and very inflamed, while the untreated wound appeared to be healing well. On the 14th day both wounds were discharging pus, but the treated wound was clearly in a worse state than the untreated one. On the 24th day the

animal was killed and both wounds opened, the untreated one contained some pus, but appeared to be healing, while the treated wound contained much pus, showed considerable necrosis of skin and muscle, and was surrounded by swollen and inflamed tissue

### *Inactivation of notatin in the body*

Notatin loses its antibacterial action in the body. Large doses have been given to rabbits, both intravenously and subcutaneously, and samples of blood removed at varying intervals. In no case has any antibacterial activity been detectable in these samples when tested *in vitro*. Also the antibacterial activity *in vitro* is inhibited in the presence of whole blood, defibrinated blood or serum, this observation has been confirmed by Mr Freeman. Heat treatment of serum destroys its power of inactivation.

### *Effect on diuresis*

Two groups of eight male rats were given known volumes of water by stomach tube, after being deprived of food and water overnight. The animals in one group received a subcutaneous injection of notatin (0.3 mg/kg) at the time of administration of the water, and the urine from both groups was collected for 30 hours. A week later the experiment was repeated with the same rats, the two groups being crossed over. The results were expressed as a mean percentage of the total water given which had been excreted as urine at various times after dosing, all sixteen animals being included in both treated and control groups. The results are given in Table II.

TABLE II  
EFFECT ON DIURESIS IN RATS

Time after giving water and notatin	Percentage of total water excreted	
	Controls	Notatin treated
2 hours	51	40
4 hours	69	53
7 hours	72	54
22 hours	99	62
30 hours	107	66

Similar experiments were carried out with varying doses of notatin, and they indicated that single doses of 0.1 mg/kg and upwards have a powerful anti-diuretic action, resulting in the retention in the body of a considerable portion of the water administered for a period of at least 30 hours.

### *Effect on smooth muscle*

Notatin causes contraction of rabbit intestine and virgin guinea-pig uterus *in vitro*, concentrations of 1 in 40,000 down to 1 in 200,000 cause fairly powerful

contractions of the latter muscle, this observation has been confirmed by Miss Chapman. Dr Wien has shown that notatin produces vasoconstriction in the perfused rabbit ear, in a concentration of 1 m 1,000

### *Antibacterial activity in vivo*

Tests of antibacterial activity *in vivo* were carried out in male mice of about 20 g weight. A suspension of the organism was injected intraperitoneally, and this was followed immediately by an injection of notatin. In some cases this was the only dose of notatin, and in others it was the first of a series of doses at regular intervals.

Experiments were carried out with three organisms lethal to mice: *Streptococcus haemolyticus* (Richards), *Staphylococcus aureus* (CN 59), and a virulent strain of salmonella isolated from laboratory mice during an epidemic.

Notatin was administered in a variety of ways: subcutaneously, intravenously, and intraperitoneally, as a single dose, and as a series of doses at regular intervals, in doses up to 10 mg. per kg. body weight, the maximum tolerated dose, and in some cases with simultaneous injection of glucose. In most of the experiments penicillin or sulphonamides were used as positive control treatments.

The results obtained showed clearly that notatin had no therapeutic activity in these experiments. In view of the negative results, detailed protocols of the experiments are not given. Mr Standfast also carried out an extensive series of experiments on mice infected with a strain of haemolytic streptococcus, with similar negative results.

Some experiments were also carried out with a strain of *Staphylococcus aureus* (Mrs Fox) which is not lethal to mice, the usual criterion of therapeutic action—i.e., survival of animals which would otherwise have died—could not, therefore, be used. It was found that if a suitable infecting dose (of the order of 100 million organisms) was given by intraperitoneal injection, a number of small abscesses developed within a few days in the peritoneal cavity, and could be enumerated on autopsy, and an attempt was made to use the number of abscesses as a criterion of therapeutic effect.

A number of mice were given equal infecting doses, after which some groups were given subcutaneous injections of notatin or another drug, either as a single dose or as a series of doses spread over 24 hours, and other groups were left untreated to serve as controls. At a definite interval after injection (usually 72 hours) the mice were killed and autopsied, and the abscesses visible within the peritoneal cavity enumerated, no account being taken of variation in size. The counts varied from 0 to 9 abscesses per mouse.

The results were difficult to interpret. While the animals receiving the various drug treatments (notatin, penicillin, or sulphathiazole) generally showed lower abscess counts than the untreated controls, and the difference was often statistically significant, yet the degree of lowering was erratic and bore little relation to either the drug given or the amount. A given dose level of a given drug would produce a large and significant lowering of the count in one experiment, and have no effect in the next, while a larger or smaller dose of the same drug would behave in the opposite manner.

On the whole notatin caused a greater reduction of the abscess count than did penicillin, in contradistinction to the relative therapeutic activities in the other

*in vivo* tests (including those using *Staphylococcus aureus* C N 59) in all of which penicillin was effective while notatin was inactive. Certain aspects of the technique were investigated as possible sources of erratic variation in the counts, e.g., settling of the bacterial suspensions, and fatigue of the operator during the counting of the abscesses but the erratic results could not be explained on these lines.

Some twenty experiments were performed in all, for reasons of space, the results cannot be given *in extenso*, but as the method has not been described previously, so far as we are aware, the protocol of one experiment is given in Table III. In this experiment both the mice treated with notatin and those treated with penicillin showed significantly lower counts than the controls.

TABLE III  
EFFECT ON INTRAPERITONEAL ABSCESES IN MICE

Organism	<i>Staphylococcus aureus</i> (Mrs Fox)
Infection	380 million organisms per mouse.
Treatment	Total dose of 0.1 mg. per kg. notatin or 1,000 Oxford units per kg. penicillin, divided into two equal doses at 0 and 4 hours after injection
Counted	75 hours after infection

Treatment	No. of Mice	Individual counts of intraperitoneal abscesses	Mean count
Controls	10	6, 6, 9, 6, 6, 5, 3, 6, 4, 6	5.7
Notatin	10	1, 0, 3, 2, 4, 4, 5, 4, 3, 4	3.0
Penicillin	10	5, 5, 5, 4, 5, 2, 4, 4, 2, 1	3.7

## DISCUSSION

It has been shown that notatin is highly toxic to animals. When given intravenously it interferes with the oxygen-carrying function of the blood by converting haemoglobin to methaemoglobin, and also causes gross oedema of the lungs. The two conditions proceed simultaneously, and death by asphyxia occurs rapidly. In the rat the blood pigment formed has been identified as methaemoglobin and this has also been found in the etherized rabbit, in the unanaesthetized rabbit the blood darkens, and loses the power of taking up oxygen, but the pigment formed does not appear to be methaemoglobin.

Notatin converts the haemoglobin of rabbit's blood to methaemoglobin *in vitro* only if excess glucose is added. It therefore seems probable that the formation of methaemoglobin is linked with the oxidation of glucose to gluconic acid by atmospheric oxygen, a process which is known to be catalysed by notatin, a reaction of the following kind may occur



Large subcutaneous doses of notatin cause death after several hours. There are no symptoms of asphyxia and the haemoglobin is unaffected, but the blood



sugar (at least in rabbits) rises considerably, at the expense of the liver glycogen. The toxicity is about three times greater when given subcutaneously than it is when given intravenously, together with the difference in symptoms, this leads to the conclusion that the mode of death is different in the two cases. The cause of death after subcutaneous injection is not clear, although it appears to be linked with the rise in blood sugar. It was not found possible to produce the symptoms of intravenous administration by giving notatin subcutaneously, however large the dose.

Sub-lethal subcutaneous doses given daily for a long period have no apparent systemic toxic effect. Subcutaneous injection or local application produce marked tissue damage, including oedema, haemorrhage, and necrosis. The only marked systemic effect of non-lethal doses which has been observed is a powerful anti-diuretic action: small subcutaneous doses cause retention of water in the body for 30 hours or more. The anti-diuretic action and characteristic severe oedema produced by notatin suggest that it is a powerful capillary poison.

No definite evidence has been obtained of any antibacterial activity *in vivo*, mice were not protected from lethal doses of streptococci, staphylococci, or salmonella by notatin given either intravenously or subcutaneously. Since the antibacterial action of notatin *in vitro* in the presence of glucose is due to the hydrogen peroxide liberated, it is easy to understand its inactivity *in vivo*, as any hydrogen peroxide formed would be immediately destroyed by the catalase universally present in the blood and tissues.

The significance of the staphylococcal abscess experiments is doubtful. In many cases notatin reduced the number of intraperitoneal abscesses formed after injection of a non-lethal strain of staphylococcus in mice, other drugs of known antibacterial efficiency (penicillin and sulphathiazole) also produced this effect. There was, however, no relationship between the size of dose and degree of effect, and the relative efficiencies of the three drugs and of various doses of them ran quite contrary to their known relative efficiencies in normal *in vivo* experiments in which survival is the criterion. The abscess experiments cannot therefore be accepted as unequivocal evidence of antibacterial activity.

#### SUMMARY

1 The purest samples of notatin yet obtained have the following acute median lethal doses

		Mg per kg body weight
<i>Mice</i>	Intraperitoneal	3
	Subcutaneous	4.5
	Intravenous	13
<i>Rabbits</i>	Subcutaneous	7.5

The oral toxicity is low: doses up to 300 mg per kg have no effect.

2 Fatal intravenous doses of notatin cause rapid death by anoxia due to pulmonary oedema and loss of the oxygen-carrying capacity of the blood, in some species severe methaemoglobinemia occurs

3 Fatal subcutaneous doses of notatin cause death several hours later the cause of death is unknown, but death is associated with a progressive and marked rise in blood sugar (at least in the rabbit)

4 Local application of notatin causes marked tissue damage Severe oedema, haemorrhage, and necrosis occur

5 Single small subcutaneous doses of notatin cause retention of water in the body for periods of 30 hours or more Notatin appears to act as a powerful capillary poison

6 Notatin causes contraction of isolated smooth muscle, and produces vasoconstriction in the perfused rabbit ear

7 Notatin does not exhibit antibacterial activity *in vitro* in the presence of blood or serum

8 Notatin does not protect mice from lethal doses of streptococci, staphylococci, or salmonella

9 Notatin sometimes reduces the number of intraperitoneal abscesses formed after intraperitoneal injection of a non-lethal strain of *Staphylococcus aureus* in mice, but it is doubtful whether this indicates any antibacterial action

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## CHANGES IN *CIS*- AND *TRANS*- STILBAMIDINE SOLUTIONS ON EXPOSURE TO LIGHT

BY

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The changes which *trans*- 4 4'-diamidinostilbene (stilbamidine) undergoes in aqueous solutions exposed to light have recently received much attention. Fulton and Yorke (1942) demonstrated experimentally the greatly increased toxicity of exposed solutions for mice. Barber, Slack, and Wien (1943) concluded that the main product of change was 4 4'-diamidinophenylbenzylcarbinol produced by the addition of water to the ethylenic double bond in the parent substance. The saturation of this double bond was confirmed spectrographically by Goodwin (1943). Henry (1943) stated that one or both amidine groups were hydrolysed by prolonged exposure to diffuse daylight with accompanying formation of ammonium chloride in solution. He believed that a dimer of the parent substance was formed as a principal primary product as well as *cis*- stilbamidine.

We have investigated the changes which *cis*- and *trans*- stilbamidine undergo on exposure to light in solution by biological as well as spectrographic and chemical methods. We are indebted to Dr H J Barber of May and Baker, Ltd., for a gift of *cis*- stilbamidine and also for the purified product of irradiation of the *trans*- form.

### EXPERIMENTAL

#### *Biological*

The toxicities of fresh and exposed solutions of *cis*- and *trans*- forms in distilled water were determined in mice by intraperitoneal injection. The *cis*- form was used as sulphate dissolved in the cold to give a 0.2 per cent solution. The *trans*- sulphate is almost insoluble, and solutions of the dihydrochloride were used. Similar solutions were tested after being kept in the dark. Therapeutic experiments with these solutions were also carried out in mice infected with *T. rhodesiense*. The results obtained are recorded in Tables I and II. It will be seen that the *cis*- form, in freshly prepared solutions, is more toxic and less active therapeutically than the *trans*- isomer. Both are stable when stored in solution in the dark (confirmed spectrographically). When exposed to light the *cis*- form in the above concentration became less toxic for mice and more active

therapeutically, which is the reverse of what takes place with the *trans*- form. The reason for these changes is discussed below. It was noted that a 0.2 per cent solution of the *cis*- form began to deposit crystals on the sides of the flask within an hour after exposure, whereas a 0.05 per cent solution did not give rise to any deposit however long exposed. In the animal tests the stronger solution was administered after making a homogeneous suspension by shaking. The examination of the supernatant solution and crystalline deposit is described in the next section.

TABLE I

TOXIC EFFECTS OF FRESH AND EXPOSED SOLUTIONS OF *CIS*- AND *TRANS*- STILBAMIDINE IN MICE

D = Died in less than 1 hour after injection  
 P = Died within a few days of injection  
 S = Survived during observation period of two weeks or longer

Drug	Nature of solution	Effect of doses (mg /20 g mouse, intraperitoneally)		
		1.0	0.5	0.25
<i>Cis</i> - stilbamidine sulphate (contains 72.9% of the base)	Fresh	6D/6	4D / 2P /10* 4S /	5S/5
	7 days in dark	5D/5	8D / 2S /10	5S/5
	14 days in dark	3D/3	6D / 4S /10	5S/5
	7 days in sunlight	8D / 1P /10 1S /	1P / 14S /15	5S/5
	14 days in sunlight	5D/5	1D / 9S /10	5S/5
<i>Trans</i> - stilbamidine dihydrochloride (contains 78.3% of the base)	Fresh	1P / 9S /10	10S/10	—
	7 days in sunlight	—	5D/5	5S/5*

\* Doses not well tolerated by surviving animals.

### Chemical and spectrographic

Most spectrographic data were obtained with a Beckman photoelectric spectrophotometer (Cary and Beckman, 1941), but at the start of the investigation photographic records were obtained with a Hilger E3 spectrograph used in conjunction with a rotating sector photometer and iron-nickel arc.

It was found that the absorption spectra of *cis*- and *trans*- stilbamidine (Fig. 1) bore the same relationship to one another as those of the parent stilbenes.

(Smakula and Wasserman, 1931), thus indicating the purity of the specimens employed. In the *cis*- compound the  $\epsilon$  value is reduced and the position of  $\lambda$  max is shifted to shorter wavelengths (*cis*-  $\epsilon$  max 14,000,  $\lambda$  max 299 m $\mu$ , *trans*-  $\epsilon$  max 37,800,  $\lambda$  max 329 m $\mu$ )

TABLE II

RESULTS OF TREATMENT OF *T. RHODESIENSE* INFECTIONS IN MICE WITH SOLUTIONS OF *CIS*- AND *TRANS*- STILBAMIDINE

D = Died within an hour of receiving drug.

C = Cured

R = Blood free from trypanosomes but a relapse occurred

N = Blood never free from trypanosomes.

Drug	Nature of solution	Effect of doses (mg./20 g. mouse, intraperitoneally)					
		0.5	0.25	0.1	0.05	0.025	0.01
<i>Cis</i> - stilbamidine sulphate	Fresh	2D / 1R / 1C /	3R / 1C /	4R /4	3R /3	3N /3	4N /4
	7 days in sunlight	5C /5	5C /5	4C /4	2R / 3C /5	8R / 1C / 9	2N / 3R / 5
	14 days in sunlight	—	—	5C /5	4R / 1C /5	2N / 2R /5 1C /	3N / 2R / 5
<i>Trans</i> - stilbamidine dihydrochloride	Fresh	4C /4	4C /4	4C /4	3C /3	3C /3	2R / 2C /4

In view of the biological changes noted in the exposed *cis*- solutions the solid deposit was separated by centrifugation and examined separately from the supernatant liquid. The absorption spectrum of the latter was identical with the irradiation product of *trans*- stilbamidine (Fig 1), and its toxicity for mice was of the same order. The solid which had separated was identified as *trans*-stilbamidine sulphate by its insolubility in water and other solvents, by its fluorescence, solubility in hydrochloric acid and characteristic absorption spectrum in that medium, as well as by analysis (Found C, 52.40, H, 4.99, N, 15.42, S, 8.41. Calc for  $C_{16}H_{14}N_4H_2SO_4$  C, 53.0, H, 4.98, N, 15.47, S, 8.84 per cent). The fact that the solid *trans*- sulphate is produced from a 0.2 per cent irradiated solution of the *cis*- isomer gives an explanation of the changes which occur in biological properties (Tables I and II). The therapeutically more active *trans*- form which is deposited as solid remains unaffected by light (Fulton and Goodwin, 1945), whereas the relatively small amount of *trans*- form remaining in solution is converted to the saturated product as shown by spectrographic evidence.

In more dilute solutions (0.05 per cent) of the *cis*- compound no precipitation occurred on irradiation, and an examination of the absorption spectra of such solutions irradiated for varying periods of time showed that small changes took place within half an hour from the start of exposure. Even after 72 hours, however, no further change could be detected. The new absorption curve corresponded with that obtained by a combination representing 89 per cent of the *cis*- and 11 per

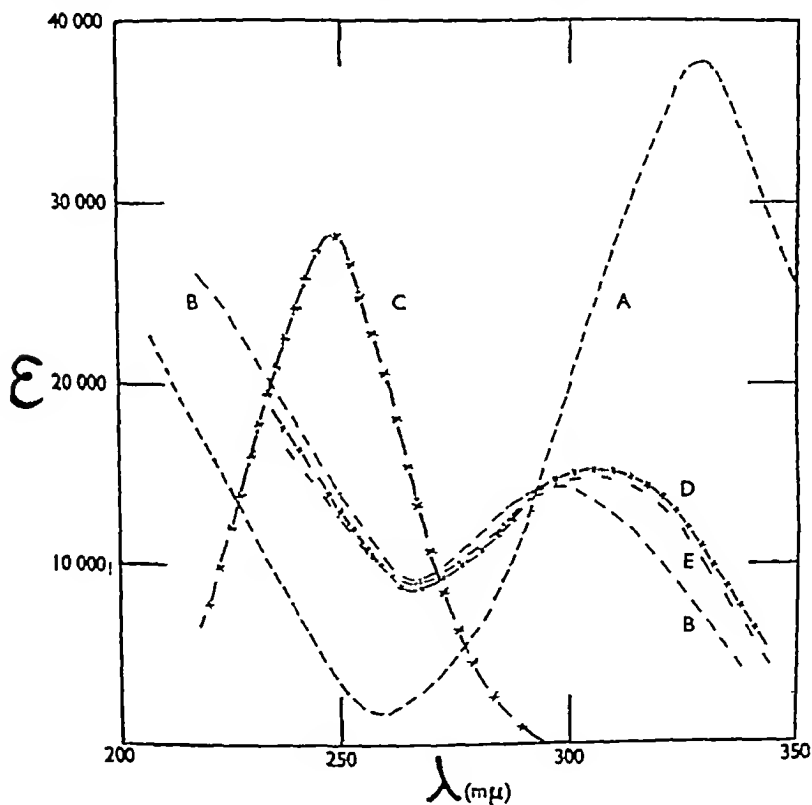


FIG. 1—Absorption spectra of *cis*- and *trans*-stilbamidine. A --- *trans*-stilbamidine, fresh, B --- *cis*-stilbamidine, fresh, C - x - x product of irradiation of *trans*-stilbamidine, D - x - x - *cis*-stilbamidine (0.01 per cent) exposed to UV light, E computed curve for a 9:1 mixture of *cis*- and *trans*-stilbamidine.

cent of the *trans*- isomers. It was difficult to state definitely that the saturated product was completely absent in the solutions. A computed curve for 85 per cent *cis*-, 10 per cent *trans*- and up to 5 per cent of the saturated compound was not definitely distinguishable from the 89/11 per cent *cis*- *trans*- curve. The correct conclusion to be drawn is that if the saturated product was present in solution it was in a concentration of less than 5 per cent of the dissolved substances.

Pure *trans*- solutions undergo photochemical change so quickly with production of the saturated compound that any tendency to *cis-trans*- equilibrium is not observed. This fact, combined with the results already considered, points to the conclusion that *cis-trans*- rotation is the first photochemical change and that it is the *trans*- compound which undergoes the change involving saturation of the double bond.

Why dilute solutions of the *cis*- form give rise to a relatively stable equilibrium with the *trans*- isomer without the latter being converted to the saturated substance in any appreciable amount, with resultant disturbance of such equilibrium, cannot be easily answered. Repeated experiments have, however, given similar results. Here, no precipitation of the *trans*- sulphate occurs as in the case of the stronger solution. If it be assumed that the *trans*- form is stabilized in some way, the results obtained on irradiation of concentrated and dilute solutions of the *cis*- compound are not necessarily contradictory. The strong *cis*- solution could proceed to equilibrium with formation of 11 per cent of the *trans*- compound when the solubility product of the *trans*- sulphate would be exceeded, precipitation of the latter would disturb the equilibrium. If no stabilization of the *trans*- form occurred, the solubility product of its sulphate would not be exceeded on account of the formation of the saturated form.

It is very probable that the formation of the "saturated" product by irradiating aqueous solutions of *trans*- stilbamidine is due to collisions between activated *trans*- molecules. The high absorption coefficient of this compound indicates that a large proportion of the molecules present in an irradiated solution may be activated. In a solution in which the *trans*- isomer is the only solute, the chance of collisions between activated molecules will thus be high, permitting rapid formation of the saturated compound. In a dilute solution containing a mixture of 89 per cent *cis*- and 11 per cent *trans*- stilbamidine an activated *trans*- molecule is much more likely to collide with a *cis*- molecule than with another *trans*- molecule. Deactivation consequent upon such collisions may well be the explanation of the "stabilizing" of the *trans*- compound in such a solution resulting in an apparent equilibrium. In a concentrated *cis*- solution the formation of *trans*- proceeds almost to completion owing to its removal from solution as the insoluble sulphate. Towards the end of the photochemical reaction small amounts of both isomers probably remain in solution, but now the ratio of *cis*- to *trans*- is much less than in the dilute solution discussed above. Chances of collisions between activated *trans*- molecules with formation of the saturated compound are much greater. Small amounts of this substance were in fact detected (Table III).

*Trans*- stilbamidine base in isobutyl alcohol distilled over calcium metal had the same absorption spectrum in fresh preparations as an aqueous solution of the salt. Both solutions underwent the same changes on exposure to light, the former much more slowly than the latter. In view of the slight solubility of the

base in isobutyl alcohol and the small amount exposed it was difficult to exclude the rôle which traces of water may have played in formation of the saturated compound. The same change into the saturated product occurred in the absence of oxygen when stilbamidine in boiled distilled water in a sealed tube filled with nitrogen gas was exposed to light. The formation of the carbinol involves the production of an asymmetric centre, but, as we expected, under the conditions of experiment, both *cis*- and *trans*- solutions after exposure remained optically inactive.

Table III shows the amount of each substance present following irradiation of a 0.2 per cent solution of *cis*- stilbamidine sulphate under different conditions

TABLE III

PRODUCTS OF IRRADIATION OF 10 MG *CIS*-STILBAMIDINE IN 0.2 PER CENT SOLUTION

	Time of irradiation			
	7 days sunlight	7 days sunlight	12 hrs. u v lamp	14 days sunlight
<i>Cis</i> - form unchanged	Not estimated	Not estimated	1.1 mg	0.9 mg
Saturated compound	3.6 mg	2.8 mg	2.4 "	1.5 "
<i>Trans</i> - form as solid	4.3 "	6.4 "	5.4 "	6.4 "
Percentage recovery	[79]	[92]	89	87

Contrary to the findings of Henry (1943) we have not been able to detect the presence of ammonia in exposed solutions in any significant amount. Our method consisted in aspirating these solutions into Nessler's reagent both before and after alkalization with potassium carbonate. Only the minutest traces were detected and we therefore concluded that under our conditions of experiment hydrolysis to amide did not occur, or only to a negligible degree, and in support of this fact precipitation of solid from exposed solutions of *trans*-stilbamidine was never detected. These conclusions were confirmed by spectrographic data. Thus, the irradiated product, whatever its constitution, has an  $\epsilon$  value much higher than that of diphenyl ethane (amidine groups absent) and suggests that these groups remain intact after irradiation. Further, irradiated *trans*-stilbamidine gives an absorption spectrum with molecular extinction coefficient identical with that of the purified compound isolated from the irradiated solution which shows that complete conversion to the latter occurs. In irradiation experiments with the *cis*- compound only 80-90 per cent of the original material could be accounted for, but as very small quantities were used it is probable that the manipulations involved accounted for the loss. The measurement of  $\epsilon$  would not allow us to distinguish between the parent substance and a dimer, if such were formed, with amidine groups intact. However,



from an examination of the position of the absorption bands and toxicities for mice of *p*-tolamidine ( $\lambda$  max 241 m $\mu$  4 mg tolerated per 20 g mouse i.p.), 4,4'-diamidinodiphenylethane ( $\lambda$  max 245 m $\mu$  1.0 mg tolerated) and the irradiated product ( $\lambda$  max 251 m $\mu$  0.1-0.2 mg tolerated) it appears that a cleavage or simple reduction of stilbamidine at the ethylenic double bond has not taken place

#### SUMMARY

An examination has been made by biological and spectrographic methods of the changes which occur in aqueous solutions of *cis*- and *trans*-stilbamidine when exposed to light. From experimental data we conclude that the change *cis*→*trans* occurs previous to formation of the saturated product of irradiation. No evidence of the reverse *trans*→*cis* change was obtained. Under our experimental conditions no conversion of the parent substances to amides occurred. Some observations have been made on the nature of the irradiation product formed.

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# EXPERIMENTAL DIPHTHEROID INFECTIONS OF THE RABBIT'S EYE AND THEIR TREATMENT

BY

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In the course of investigations of trauma produced by various toxic agents in the rabbit's eye, it was found that infection usually occurred. The organism most frequently recovered was a diphtheroid of the Hofmann group, and evidence was obtained that the infection with this organism materially contributed to the severity of the ensuing lesions.

It became of interest to determine (1) the methods by which infective lesions of the eye could be produced by this organism, and (2) the effect of various antiseptic and chemotherapeutic agents upon this infection.

## MATERIALS

### *Bacteriology of the organism*

The organism used in the present investigation (Ho R) is a Gram-positive bacillus which shows perfect uniformity in films from cultures and appears as a rod, sometimes so short that it might be mistaken for a coccus of a slightly ovoid shape. In the Albert stain (modification of Neisser's method) many metachromatic granules are seen, but their size and arrangement are much less regular than those in the diphtheria bacillus. Films made from the purulent corneal lesion always show the organism in a definite bacillary form, sometimes even considerably elongated. The growth of the organism on blood agar is rapid and luxuriant, large, round, elevated colonies of moist, smooth surface and a definite white or slightly yellow colour are formed, this cultural appearance is almost identical with that of staphylococcus aureus, from which it could hardly be differentiated without further study. The growth of Ho R on ordinary agar is equally abundant but is apt to be unreliable, very small inocula may fail to grow at all. The growth in ordinary broth is poor and slightly granular. Suspensions in saline, if made from blood agar, show a similar granular appearance, if made from ordinary agar, however, a perfectly homogeneous suspension can be obtained.

No attempt was made to relate the organism to any of the animal-pathogenic diphtheroids, but in one test on mice it was found that intraperitoneal injection of half a loopful was lethal within 24 hours with the production of bacteraemia, while 1/100th of this dose produced no effect.

It is worth mentioning that several times in the course of our experiments the organisms isolated from the infected eyes had been transformed into a variant with the features of *C. xerosis* which grows in characteristically small colonies.

*Therapeutic substances*

The following substances were used

- (1) Penicillin a solution of 500 units per ml
  - (2) Sodium sulphacetamide 30 g/100 ml, used in combination with a detergent.
  - (3) Marfanil (*p*-aminomethyl benzene sulphonamide) 10 and 30 g/100 ml Solutions prepared by dissolving marfanil hydrochloride in saline and neutralizing to pH 7.0
  - (4) V335 (*p* methylsulphonyl benzylamine) 10 and 30 g/100 ml The hydrochloride was dissolved in saline and neutralized to pH 7.0
  - (5) V187 (*p* methylsulphonyl benzamidine) 10 g/100 ml The hydrochloride was dissolved in saline, giving a solution of pH 6.0, which was not neutralized, as the free amidine base is liable to be precipitated
- V335 and V187 are new chemotherapeutic substances described by Evans, Fuller, and Walker (1944)
- (6) Proflavine 0.1 g./100 ml A buffered solution of pH 6.3 was made up from isoflav (Boots)
  - (7) In two experiments a detergent, 0.1 g/100 ml duponal (sodium lauryl sulphate), was added to the solutions of sodium sulphacetamide and marfanil

## METHODS

*In vitro tests of drugs on Ho.R*

For testing bacteriostatic and bactericidal efficacy in one and the same operation the following simple technique was devised. Small test tubes were used, receiving 1 ml. of the drug solution in serial aqueous dilutions. To each dilution and a saline control was added a drop of a dense suspension of Ho R (50–60,000 million per ml) so that the number of organisms in each tube was about 1,500 million. The tubes were kept at room temperature (about 18°C), shaken from time to time, and tested at intervals by inoculation on blood agar plates. This inoculation was done by a method which made possible the determination of both bacteriostatic and bactericidal effects. The plates were thoroughly dried before inoculation and divided into 6–8 horizontal strips, each strip being reserved for the testing of one tube. A loopful was taken from the bottom part of each test tube without shaking and seeded at one end of an agar strip on a circular area 10–15 mm. in diameter (primary inoculation area). The drop was allowed to soak into the medium, while being spread over this small circle, within 5–10 seconds. When all the primary areas were inoculated in this way, the organisms deposited on each of them were spread over the remaining part of the agar strip: the loop was stroked several times over the whole of the primary area and then, without retouching it, stroked in as many parallel lines as possible over the so far untouched length of the strip (secondary inoculation area). It is obvious that the primary areas contain the organisms together with the drug, though in a concentration which is probably well below that in the corresponding test tube, owing to the diffusion of the fluid into the agar. The secondary areas on the other hand contain the organisms freed from the drug, which has seeped away before the bacterial inoculum has been picked up from the dry surface of the primary inoculation area. The correctness of this assumption was borne out by the appearance of the plates after 24 hours' incubation: a bacteriostatic effect was shown by absence of growth in the primary area and abundant growth in the secondary area, absence of growth in both zones indicated a bactericidal effect in the test tube, whereas equally full growth in both areas proved the lack of any bacteriostatic or bactericidal effect.

*In vitro* tests with some of the drugs on Ho R gave the following results. Penicillin was bacteriostatic in the smallest concentration used, viz., 28 units per ml, at 226 units per ml it was strongly but not completely bactericidal in

24, but not in 4 hours. at 113 units per ml it was not bactericidal in 24 hours Sulphacetamide was bacteriostatic at 0.75 g/100 ml, but not bactericidal at 30 g/100 ml even after 48 hours Marfanil had no bacteriostatic or bactericidal effect whatever at a concentration of 1 g/100 ml, at 10 g/100 ml it was completely bacteriostatic and killed the organisms in 48, but not in 24 hours Proflavine had no bacteriostatic effect at all, but within 24 hours, though not in 4 hours, it killed the organisms at 0.4 g/100 ml and still showed at 0.1 g/100 ml a marked though incomplete bactericidal effect

### *Animal Experiments*

(1) *Intracorneal injections*—Intracorneal injections were performed by a method previously described (Robson and Scott, 1943) When a suspension containing 25 million organisms per ml was used, an extensive abscess of the cornea developed in 48 hours, ending in perforation With a suspension containing fewer organisms the severity of the lesions showed great variation in different animals, occasionally a severe lesion resulted from injection of a suspension containing so few as 2,500 organisms per ml., while in other animals the injection of a suspension containing 2.5 million per ml produced only a mild lesion Consequently, this method was considered unsatisfactory for the investigation of therapeutic substances

(2) *Inoculation of the denuded cornea*.—In these experiments the epithelium was removed from an area about 6×6 mm in the centre of the cornea. In the absence of deliberate infection the denuded area becomes epithelialized within 48 hours Inoculation of the conjunctival sac with a few drops of a suspension containing 2,500 million organisms per ml. was always followed by infection of the denuded area, which delayed epithelialization In some animals severe ulcers developed, and in others only a mild infiltrative process, healing within a week. When this suspension was diluted 100 times there was no appreciable delay in the process of healing This method also was considered unsuitable for therapeutic investigations

(3) *Deep scarification*—The method found suitable for testing drugs was as follows The corneal epithelium was removed from an area 6×6 mm. and the subjacent substantia lightly scarified with a discission needle Inoculation of the conjunctival sac with the diphtheroid suspension (2,500 million per ml) was always followed by the development of a severe and progressive infection running a fairly typical course In 24 hours the area staining with fluoresceine was more extensive than that originally denuded, and the cornea in this area was very hazv In two to three days the lesion became a corneal abscess which spread and, in the more severe cases, involved most of the cornea by the end of the first week, in many cases perforation occurred. The accompanying conjunctivitis was always severe, with profuse discharge, and membrane formation on the palpebral conjunctiva was common

(4) *Treatment*—In all animal experiments lesions were produced in both eyes, one eye being treated and the other used as control At each treatment three drops of the drug solution were instilled into one eye, whilst the control eye received an equal amount of normal saline Treatment was started one or six hours after the production of the lesion and continued at hourly intervals for 8-11 hours on the first day, for 13 hours on the second day and for 8 hours on subsequent days The duration of treatment was from 7 to 10 days

Cultures were taken from the conjunctival sacs in the mornings always before the first treatment

The results were assessed numerically according to an arbitrary scale

1=slight

3=severe, and

5=very severe with perforation

Intermediate figures were used for lesions of intermediate severity

## RESULTS

These are given in Table I

### *Deep scarification (diphtheroid infection)*

It will be seen that penicillin, 30 g /100 ml marfanil, and 30 g /100 ml V335 all had a very beneficial effect when the treatment was started an hour after infection, at 6 hours the effect was slight but definite

Marfanil and V335 were appreciably less effective in 10 g /100 ml solutions, and at 6 hours these weaker solutions had no significant effect even, in the case of marfanil, when combined with the detergent V187 was without appreciable effect.

TABLE I  
TREATMENT OF LESION PRODUCED BY INOCULATION OF SCARIFIED CORNEA  
WITH DIPHThEROID

Treatment	Interval between inoculation and first treatment (Hours)	Number of animals	Assessment of final corneal lesion (Mean values on arbitrary scale)		Difference between treated and control eyes
			Treated eyes	Control eyes	
Marfanil {	30 g /100 ml	5	1.4	4.0	2.6
	10 " "	3	3.4	5.0	1.6
	30 " "	3	2.5	3.9	1.4
	10 " "	3	4.4	4.7	0.3
	10 " "				
	+ duponal	3	4.0	4.5	0.5
V335 {	30 g /100 ml	3	1.6	5.0	3.4
	10 " "	3	2.8	4.9	2.1
	30 " "	3	3.7	5.0	1.3
	10 " "	3	4.6	4.8	0.2
V187 10 ,	1	3	4.3	4.7	0.4
Penicillin 500 U/ml {	1	6	2.2	5.0	2.8
	6	3	2.7	4.1	1.4
Proflavine 0.1 g./100 ml	1	3	3.5	5.0	1.5
Sodium sulphacetamide 30 g /100 ml + duponal	1	3	4.1	3.8	-0.3

Proflavine (0.1 g /100 ml) was found to be of definite value in these surface infections, though it had previously been found to be of little or no value in the

treatment of lesions produced by the intracorneal injection of the pneumococcus, staphylococcus aureus, and haemolytic streptococcus (Robson and Scott, 1944) Sodium sulphacetamide (30 g/100 ml) combined with the detergent, was ineffective, this is in contrast to the considerable bacteriostatic effect of this sulphonamide on Ho.R when tested *in vitro*, and at present we have no explanation of this discrepancy

### *Intracorneal injections*

Preliminary experiments with lesions produced by the intracorneal injection of the diphtheroid suggested that marfanil and V335 were without effect. But, since these lesions were technically unsatisfactory, no great significance could be attached to these results. It did seem desirable, however, to determine whether these drugs were in actual fact without value in intracorneal infection in view of their proved effectiveness in superficial lesions. Consequently it was decided to determine their value in intracorneal infection produced by other organisms known to be susceptible to the action of other substances, e.g., penicillin. For this purpose, standard lesions were produced by staphylococcus aureus and pneumococcus by the technique previously described (Robson and Scott, 1943). The results given in Table II show clearly that marfanil and V335 were ineffective.

TABLE II

TREATMENT OF LESIONS PRODUCED BY INTRACORNEAL INJECTION OF ORGANISMS  
Interval between inoculation and first treatment = 1 hour

Injected organism	Treatment	Number of animals	Assessment of final corneal lesion (Mean values on arbitrary scale)		Difference between treated and control eyes
			Treated eyes	Control eyes	
Staph aureus Pneumococcus (19)	Marfanil 30 g./100 ml	4	3.0	3.3	0.3
		3	2.5	3.0	0.5
Staph aureus Staph aureus	V335 30 g./100 ml	3	2.9	2.7	-0.2
		4	3.4	3.7	0.3

### DISCUSSION

The main aim of this investigation has been to devise an open infected lesion of the rabbit's cornea suitable for the testing of antiseptic and chemotherapeutic substances. The present study has been confined to the local application of drugs.

By the technique described, a lesion of fairly uniform severity was invariably produced. Such a lesion cannot be obtained by the inoculation of the deeply scarified rabbit's cornea with the main organisms pathogenic to the human eye,

such as gonococci, pneumococci, staphylococci, and streptococci. However, the infection of the rabbit's cornea with Ho.R., although this organism may be without much importance in human pathology, provides an experimental lesion, valuable as a counterpart to human disease and suitable for the testing of chemotherapeutic substances. The infection with *Pyocyaneus* previously studied (Robson and Scott, 1942) produces a severe and reasonably uniform lesion in the rabbit, but is rather unsatisfactory because this organism is insensitive to many drugs.

In the experiments with intracorneal infections only penicillin was effective (Robson and Scott, 1943), marfanil and V335 did not influence the progress of the lesions. This observation is in agreement with the previous findings that marfanil and V335 are of little value in the treatment of systemic infections. It seems likely that these drugs either do not penetrate or are in some way inactivated, but this point requires further investigation.

#### SUMMARY

1 A technique is described for the production of open infections of the rabbit's cornea, such that drugs have direct access to the lesion. The organism used was a diphtheroid of the Hofmann group; in all cases severe destructive lesions resulted.

2 Early application of penicillin (500 U/ml), marfanil (30 g/100 ml) and V335 (*p*-methylsulphonyl benzylamine, 30 g/100 ml) had a very beneficial effect on the course of the lesions, proflavine (0.1 g/100 ml) had some effect, but sodium sulphacetamide (30 g/100 ml) and V187 (*p*-methylsulphonyl benzamide, 10 g/100 ml) had no effect.

3 Marfanil and V 335 (30 g/100 ml. solutions) had no effect on lesions produced by the intracorneal injection of *staphylococcus aureus* and *pneumococcus* (type 19).

We are greatly indebted to the "W H Ross Foundation (Scotland) for the Prevention of Blindness," who have defrayed the expenses of this investigation. British Schering, Ltd., and Messrs Boots, Ltd., very kindly supplied us with some of the drugs used.

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# THE TOXICITY OF ALKYL-BIS( $\beta$ -CHLOROETHYL)-AMINES AND OF THE PRODUCTS OF THEIR REACTION WITH WATER

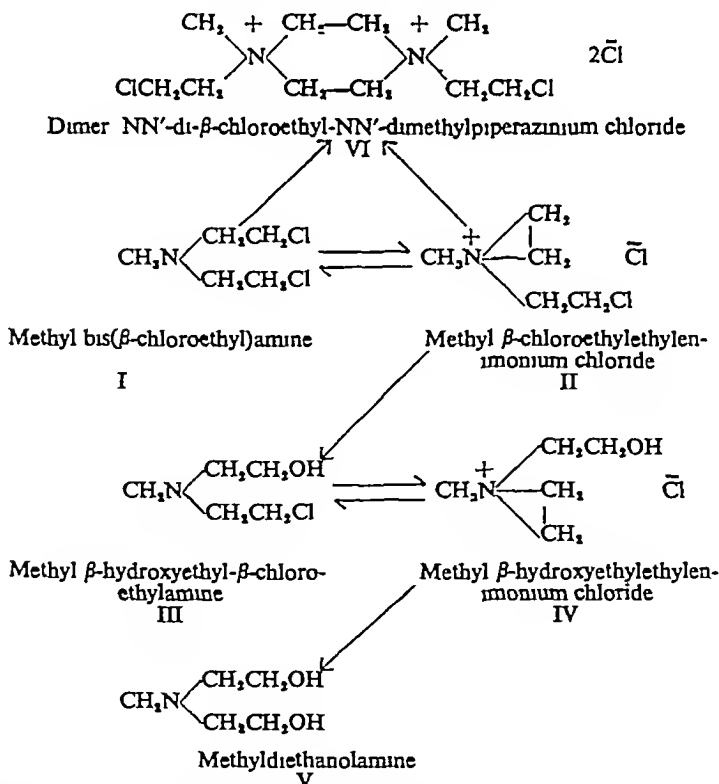
BY

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Nitrogenous vesicants or "nitrogen mustards," which are bis( $\beta$ -chloroethyl)-amine derivatives, differ from mustard gas which is bis( $\beta$ -chloroethyl)sulphide in some of their biological actions although many pharmacological effects are common to both types of compound. The pharmacology of these substances

## REACTIONS OF METHYL BIS( $\beta$ -CHLOROETHYL)AMINE IN AQUEOUS SOLUTION



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has been summarized by Gilman and Philips (1946) Two differences in behaviour are that aqueous solutions of the bis( $\beta$ -chloroethyl)amines (1) develop an acute toxicity, associated with a rapid convulsive and lethal action, and (2) remain toxic indefinitely, while aqueous solutions of mustard gas become non-toxic on standing Methyl-bis( $\beta$ -chloroethyl)amine is much more soluble in water than is bis( $\beta$ -chloroethyl)sulphide and aqueous solutions have been found by Hanby, Hartley, Powell, and Rydon to yield the products shown above (Hanby and Rydon, 1946, Hanby *et al.*, 1946) The reactions indicated are inhibited by acid, and aqueous solutions of the hydrochlorides of the vesicant bases are stable

#### *Toxicity of alkyl-bis( $\beta$ -chloroethyl)amines*

Freshly made aqueous solutions of the hydrochlorides of some of the nitrogenous vesicants were given subcutaneously to mice and rats (Table I) and orally to rabbits for determination of the LD50 When the substances were given as fresh aqueous solutions in doses near the LD50, death occurred several days after dosing, and the most usual post-mortem finding was congestion of the gut, which was filled with liquid material Small doses of the fresh nitrogenous vesicants are similar to mustard gas in their toxic action The toxicities of methyl-bis-

TABLE I

TOXICITY OF THE HYDROCHLORIDES OF NITROGEN VESICANTS ON SUBCUTANEOUS INJECTION INTO MICE AND RATS

Alkyl bis( $\beta$ -chloroethyl)amines Alkyl-N(CH <sub>2</sub> CH <sub>2</sub> Cl) <sub>2</sub> Alkyl group	Mice		Rats	
	LD50 approximate mg./kg	Number of mice used	LD50 approximate mg./kg.	Number of rats used
Methyl	4	36	2	12
Ethyl	1	40	1	12
Propyl	0.5	19	0.5	16
isopropyl	0.5	24	2	8
$\beta$ -chloroethyl	2	16	2	12

( $\beta$ -chloroethyl)amine and  $\beta\beta'\beta''$ -trichloroethylamine are of the same order as the toxicity of mustard gas (between 2 and 5 mg per kg body weight) Ethyl-bis( $\beta$ -chloroethyl)amine and propyl-bis( $\beta$ -chloroethyl)amine are possibly more toxic (with LD50 of 0.5 to 1 mg per kg body weight) Lethal doses of fresh aqueous solutions of the nitrogen mustards or of their hydrochlorides cause mice or rabbits to salivate after a short time and to die several hours or days afterwards Oral administration gave less definite results, but the lethal doses of methyl-bis( $\beta$ -chloroethyl)amine varied from 3 to 6 mg per kg for rabbits to 10 mg per kg for rats and mice Ethyl-bis( $\beta$ -chloroethyl)amine and  $\beta\beta'\beta''$ -trichloroethylamine were lethal to rabbits in doses of 2 to 6 mg per kg *per os*

Death occurred after 3 to 17 days, during which diarrhoea and loss of weight occurred. At death congestion of the stomach and intestines was usually present.

*The lethal action of the reaction products of nitrogen mustards with water*

Alkyl-bis( $\beta$ -chloroethyl)amines react with water in neutral solution to produce toxic compounds with different pharmacological properties. Hanby *et al* (1946) showed that the first reaction products include ethylenimonium compounds (as formula II). Other reactions lead to the formation of dimers (VI), hydroxyethylchloroethylamines (III), and diethanolamines (V), but except for the hydroxyethylchloroethylamines these substances are not toxic. The pharmacology has been most fully investigated with methyl-bis( $\beta$ -chloroethyl)amine, but substances with similar properties are produced from the other dichloroethylamines.

TABLE II

SURVIVAL TIMES OF MICE RECEIVING SUBCUTANEOUS INJECTIONS OF 1 G /100 ML. SOLUTIONS OF METHYL BIS( $\beta$ -CHLOROETHYL)AMINE (10 ML. PER KG.) AT ROOM TEMPERATURE (15°)

Age of solutions	Survival times with		
	aqueous solutions (final pH 4.5)	solutions in 3%NaHCO <sub>3</sub>	solutions of the hydrochloride
Hours			
2	2½ hours	—	2½ hours
6	12 "	—	—
18	35 mins	14 mins	—
24	40 "	16 "	—
48	17 "	16 "	3 hours
72	19 "	20 "	2½ hours
96	18 "	6-12 hrs.	100 min
Weeks			
1	18 "	failed to kill	125 min
2	19 "	—	110 min
3	20 "	—	55 min.
4	18 "	—	—
5	21 "	—	—
8	22 "	—	—
12	19 "	—	—

Methyl-bis( $\beta$ -chloroethyl)amine is soluble to the extent of 1 g in 100 ml water and if a solution is kept it becomes more acutely toxic, i.e., the same amount of material injected into mice kills them in less time (Table II). Subcutaneous injection (10 ml per kg) into mice of a 1 g /100 ml solution, which has been kept for two days at room temperature and has attained equilibrium, causes death in about 16 minutes. With higher doses mice die more rapidly (e.g., in 10 minutes with 200 mg per kg and in about 6 minutes with 400 mg per kg) and with lower doses more slowly. The variations in survival times of mice are indicated in Table VI. In Figs 1 and 2 and Tables II to V the survival times are mean times for 2 or 4 mice injected with the same preparation. If the survival time is plotted against the dose injected (in 10 ml of neutral solution per kg

body weight) on a logarithmic scale a straight line is obtained (Fig 1) This simple relationship between the logarithm of the dose and the survival time makes it possible to estimate the amount of the convulsive substance in solutions

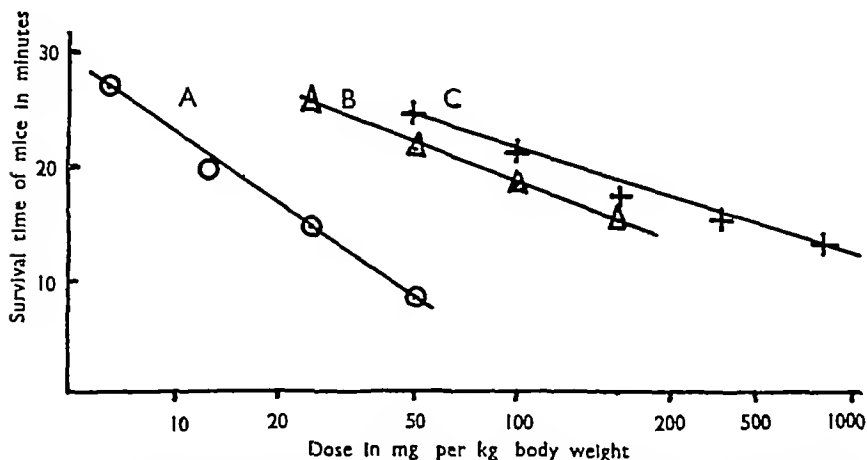


FIG 1—Relationship between dose and survival time of mice injected with  
A—Methyl- $\beta$ -chloroethyl- $\beta$ -hydroxyethylamine after one hour in neutral solution.  
B—Methyl bis( $\beta$ -chloroethyl)amine (1%) after four days in aqueous solution  
C—Methyl bis( $\beta$ -chloroethyl)amine (8%) after four days in aqueous solution

if injections are made under standardized conditions The survival time is longer if the injected solution is acid (below pH4) or if the mice are cold If the drug is injected into cold mice it has a hypnotic action and death occurs after several hours The rate at which the vesicant base is transformed into the convulsive

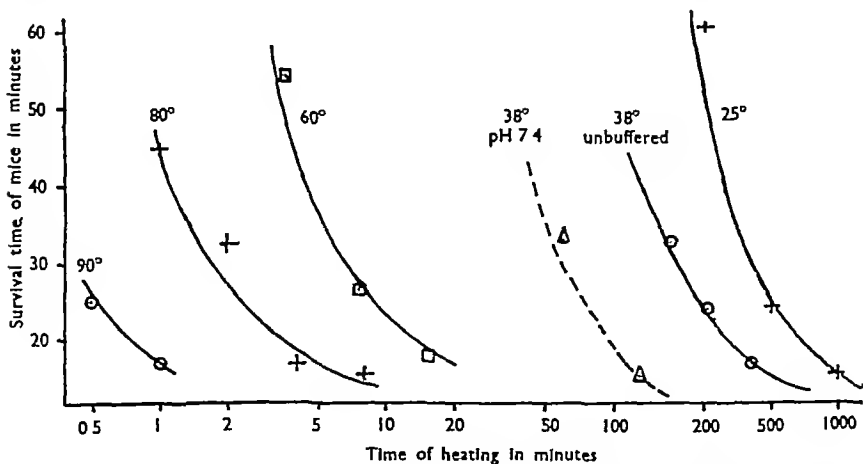


FIG 2—The relationship between the survival time of mice injected with 1% methyl-bis( $\beta$ -chloroethyl)amine in aqueous solution and the time of heating the solution at different temperatures

derivative was followed at different temperatures by determination of the survival times of mice injected with the solutions. The results (Fig 2) show that the change is more rapid at higher temperatures. The  $Q_{10}$  for the reaction was calculated from the data as 2.5 and the corresponding heat of activation as 21,000 calories. In these experiments it was noticed that when an aqueous solution (1 g /100 ml) of methyl-bis( $\beta$ -chloroethyl)amine was heated the liquid became cloudy, presumably owing to the lower solubility of the base at higher temperatures, the cloudiness however disappeared rapidly, the solution becoming clear owing to the formation of easily soluble products. The formation of the acute toxic agent is also more rapid in neutral solution (pH 7.4 buffered with  $\text{NaHCO}_3$  and  $\text{CO}_2$ ) than in the acid solution (pH 4.5) produced by the reaction of methyl-bis( $\beta$ -chloroethyl)amine with water. Fig 2 shows the reaction at  $38^\circ$  for buffered and unbuffered solutions. The convulsive substance is, however, unstable in neutral or alkaline solution. Other data showing the effect of variations in acidity on the action of the acute toxic agent are given in Table III.

TABLE III

EFFECT OF pH AT TIME OF INJECTION ON SURVIVAL TIMES OF MICE INJECTED WITH OLD SOLUTIONS OF METHYL BIS( $\beta$ -CHLOROETHYL)AMINE  
Doses as equivalent of original amine

pH	Survival time of mice	
	50 mg. per kg	200 mg. per kg.
3.6 with citric acid (M/15 buffer)	Failed to kill	12 hours
4.5 with water	43 mins	20 mins
6.0 with $\text{Na}_2\text{HPO}_4$ (M/15 buffer)	22 "	14 "
7.0 " " " "	20 "	16 "
8.2 " " " "	24 "	15 "

TABLE IV

SURVIVAL TIMES OF MICE INJECTED SUBCUTANEOUSLY WITH VARYING DOSES OF DIFFERENT CONCENTRATIONS OF FOUR-DAY-OLD SOLUTIONS OF METHYL BIS( $\beta$ -CHLOROETHYL)AMINE IN WATER

Original concentration of solution (mg./100 ml)	Survival times in minutes after injection of doses (mg./kg.)							Dose killing mice in 20 min. mg./kg
	500	250	200	125	100	50	25	
10.0	18	22	—	29	—	—	—	350
5.0	11	15	—	26	—	—	—	170
1.0	—	—	12	—	17	23	—	70
0.5	—	—	10	—	16	24	60	70

The relative amount of convulsive material formed from methyl-bis( $\beta$ -chloroethyl)amine varies with the original concentration, being less with high concentrations (Table IV), possibly because of the formation of the dimer and of acid limiting the hydrolysing reactions.

Subcutaneous injection of the acutely toxic material rapidly produced partial paralysis combined with incoordination of movements and a kinetic tremor. After a few minutes mice have extremely violent convulsions and die. Post-mortem examination of mice dying after such convulsions has not revealed any typical changes.

While the amount of the acute toxic agent can be assayed by subcutaneous injection into mice, the amount of the original base can be estimated by its toxic effect when given orally to rats. If a mixture of the nitrogen mustard and the acute toxic substances are given subcutaneously to mice, the mice die rapidly before the nitrogen mustard itself can cause death. If the mixture is given orally to rats the absorption is so slow that the acute toxic material has very little action and the rats die from the effect of the nitrogen mustard.

The acute toxic material was not precipitated by addition of 20 volumes of acetone or of excess bromine, both reagents precipitated non-toxic material, probably including polymerized derivatives. The acute toxic material was precipitated by phosphotungstic acid and could be recovered from this precipitate by cautious treatment with barium hydroxide.

#### *The nature of the acutely toxic material*

From a solution of the reaction products of methyl-bis( $\beta$ -chloroethyl)amine with water a number of products have been isolated (Hanby and Rydon, 1946). Of these the dimer (VI) was found to be non-toxic. Mice were injected with 500 mg per kg of the fresh solution and of solutions kept for 1, 2, 4, 24, and 48 hours without suffering any obvious ill effects. Other related substances which were found to be non-toxic at the same dosage level when injected as fresh solutions or as 24-hour-old solutions included dimethyl-bis( $\beta$ -chloroethyl)-ammonium iodide, N-hydroxyethyl-morpholine methiodide, N-methyl-morpholine hydrochloride,  $\beta$ -chloroethylamine and  $\beta$ -chloroethylmethylamine.

Solutions of the half hydrolysis product (III) were acutely toxic, killing mice in short times with convulsions (Fig 1 and Table V), but as the acute lethal action did not decrease on standing in aqueous solution for 30 minutes, it is

TABLE V

THE SURVIVAL TIMES OF MICE (IN MINUTES) INJECTED WITH AQUEOUS SOLUTIONS OF SUBSTANCES WHICH CAUSE RAPID DEATH

Substance	Survival times (min) after doses (mg per kg)				
	200	100	50	20	10
Methyl- $\beta$ -hydroxyethyl- $\beta$ -chloroethylamine (III) fresh	—	14	15	31	—
The same after 15 minutes at 25°	—	11	15	25	—
The same after 30 minutes at 25°	—	15	14	24	—
N- $\beta$ -chloroethyldiethanolamine	10	13	19	23	31
N- $\beta$ -chloroethyl-N- $\beta$ -acetoxyethyl methylamine	—	23	25	50	—

probable that the actual convulsive agent is the methyl hydroxyethyl ethylenimonium chloride (IV), because the open chain compound (III) would probably cyclize rapidly on standing in aqueous solution. The concentration of the open chain form would, therefore, decrease without loss of acute toxicity. The ethylenimonium form would also be formed from the open chain form fairly quickly on injection into the body.

#### Analogous convulsive poisons

Other alkyl-bis( $\beta$ -chloroethyl)amines react with water to form substances with an acute toxic action (Table VI). Ethyl-bis( $\beta$ -chloroethyl)amine appears to be the most potent of these compounds in this respect while propyl-bis( $\beta$ -chloroethyl)amine is very much less active when allowed to react with water.

TABLE VI

THE SURVIVAL TIMES OF MICE (IN MINUTES) INJECTED WITH AQUEOUS SOLUTIONS OF VESICANTS WHICH HAD BEEN ALLOWED TO STAND UNTIL EQUILIBRIUM HAD BEEN ATTAINED

Doses given in equivalent of original vesicant

S = survived 21 days

Compound	Survival times (min.) of mice after doses below (mg 'kg.)							LD50 approx. mg./kg
	200	100	50	20	10	5	2	
$\beta\beta$ -Dichlorodiethyl sulphide		S S S S	S S S S	S S S S	—	—		>100
Methyl bis( $\beta$ -chloroethyl) amine	15 17	17 19	20 22	23 26	27 34 41 S	80 S S S		5-10
Ethyl bis( $\beta$ -chloroethyl) amine	17 14	14 14	15 12	17 18	17 24 21 S	60 S S S		5-10
Propyl bis( $\beta$ -chloroethyl) amine	84 200	800 800	800 48 hr	48 hr 48 hr	48 hr 10 days	15 days S		5
Isopropyl bis- ( $\beta$ -chloroethyl)amine	25 29	20 17	1,000 2 days	1,000 2 days	S S	S S		10-20
$\beta\beta\beta$ -Trichloroethylamine		15 19 20 23	61 100 26 28	23 20 32 40	45 3 days 3 days 3 days	3 days 4 days 4 days 5 days	5 days 6 days 13 days —	2
Fresh $\beta\beta\beta$ -Trichloroethylamine		16 17 18 18	19 19 20 21	30 27 52 38	3 days 4 days 6 days 6 days	4 days 4 days 5 days 6 days	3 days 13 days	2

$\beta\beta'\beta''$ -Trichloroethylamine differs from the other nitrogen vesicants in being rapidly lethal before it has reacted with water, this compound is only very slightly soluble in water, but reacts with water to give bis( $\beta$ -hydroxyethyl)- $\beta$ -chloroethylamine (which can give rise to an imonium form) and triethanolamine (Crane and Rydon, 1946)

### DISCUSSION

The nitrogen vesicants are extremely toxic substances and it is possible that the high toxicity is in part due to the fact that they are hpoird soluble materials of low molecular weight which will probably penetrate cells readily, having penetrated they may combine with essential cell constituents or form toxic ionized imonium salts which will be less able to pass out of the cell. There will thus be "one way traffic" of the toxic compound into cells. The toxic effects of these substances include (1) vesication, (2) haemoconcentration, (3) diarrhoea, (4) inhibition of mitosis, and (5) the acute or convulsive effect described in this paper. The vesicant action is certainly due to the parent amines and the convulsive action to the reaction products with water.

### SUMMARY

Alkyl-bis( $\beta$ -chloroethyl)amines are vesicants, more soluble in water than mustard gas, which react with water to form compounds with acute convulsive action. The convulsive compounds appear to be ethylenimonium chlorides. The reaction with water is such that equilibrium is attained, so that an aqueous solution remains toxic indefinitely.

I am indebted to the Director-General of Scientific Research (Defence), Ministry of Supply, for permission to publish this work, and to Dr H. N. Rydon for supplying many of the substances used.

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# A METHOD FOR THE EVALUATION OF ANALGESIC ACTIVITY USING RATS

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Various methods for the experimental evaluation of analgesic activity have been described. They are all based upon the change which occurs in the response of the experimental subject to a painful stimulus after dosage with an active compound. Various animal species as well as man have been used as experimental subject and a variety of stimuli employed. In the examination of new compounds for analgesic activity it is undesirable to use the human subject, and if the number of compounds is large the smaller laboratory animals, rats or mice, become the animals of choice. We have tested a number of methods in which small animals were used, but none has proved entirely satisfactory.

In the ideal method the stimulus employed should provoke a characteristic and readily observed response and the intensity of the stimulus required to elicit it should vary little from animal to animal, the method should yield reproducible results and these should be in agreement with clinical experience.

A method for analgesic assay using rats is described below which in our experience has been found to satisfy these criteria. It is a simple modification of the method of D'Amour and Smith (1941, 1943), which was itself adapted from the work of Hardy, Wolff, and Goodell (1940) in man.

## APPARATUS AND PROCEDURE

The method is based upon the reaction of the rat to a heat stimulus applied to a small area of the tail.

The apparatus can be constructed from material commonly available in the laboratory. It consists essentially of a sheet of asbestos board (e.g., uralite), 1/8 in. thick and about 4 in. square, supported horizontally and having on its upper surface two strips of the same material, 4 in. long, fixed in such a way as to leave a channel about 1/4 in. wide between them. At some point along this channel a hole 1/4 in. in diameter is drilled through the asbestos sheet and a small coil of resistance wire connected through a key to a 6-volt electrical supply fixed beneath it. The wire is of such a gauge and length that with the circuit closed it is raised to a bright red heat.

The rat under test is held in a cylindrical holder of perforated zinc clamped horizontally, its tail lies along the channel and over the hole, which must be not more than 1½ in. from its tip. When the animal has become quiet in this position the circuit is closed. After an interval the animal will withdraw its tail from the channel with a sudden and characteristic flick. This interval is timed with a stopwatch and is referred to as the *reaction time*.



Rats weighing between 120 and 160 g with clean and healthy tails are used for experiment. The heating coil is adjusted initially to such a position that a majority of the animals react at about 5 seconds. The normal reaction time of a number of rats is determined precisely, the mean of three successive determinations at 2-minute intervals being taken, and those for which it is between 4 and 6 seconds are divided into groups of a convenient size. It has been found impracticable to deal with a group of more than six rats at a time. The compound under test is then given by the chosen route and at the desired dose level, and the reaction times of the rats are thereafter determined at 15-minute intervals. Analgesia is reflected in a prolongation of the reaction time, the increase over normal in the mean reaction time of the treated animals is taken as a measure of analgesic effect, and is hereafter referred to as *effect*.

When analgesia is marked, heating may be continued until the tails are severely burned without eliciting any reaction. To avoid unnecessary damage to the tails, heating is never continued for longer than 15 seconds, and if an animal has not reacted in that time analgesia is assumed to be "complete."

In preliminary experiments we were able by this technique to demonstrate analgesic activity for morphine, codeine, and pethidine, given intraperitoneally. Aspirin and phenazone, given by this route, appeared inactive in doses approaching the median lethal, but intravenously they produced readily measurable effects at much lower doses. In view of this result, intravenous administration seems preferable when new compounds are being examined for analgesic activity for the first time and was adopted for the remainder of our experiments. Injections were made into the tail vein in the third rear to the root of the tail. Morphine, codeine, and pethidine were used as their soluble hydrochlorides, phenobarbitone as its soluble sodium salt, aspirin as its sodium salt by neutralizing the acid with N NaOH to pH 6-6.5, phenazone in aqueous solution, and hashish as described below (p. 262).

## RESULTS

One drug examined fully by the procedure described above was pethidine hydrochloride. The mean analgesic effect at varying times after single intravenous doses of 1 to 15 mg/kg is shown in Table I, where the number of rats used at each dose is also given, and plotted in Fig. 1. In computing mean values a reaction time of 15 seconds was assigned to animals showing "complete" analgesia.

TABLE I  
ANALGESIC EFFECT OF PETHIDINE

Dose mg/kg. (i.v.)	No. of rats	Mean effect in seconds at the following times after injection					
		15 mins	30 mins	45 mins	60 mins	75 mins	90 mins
15	5	10.8	10.6	4.7	2.2	1.2	0.3
10	9	8.1	6.4	2.9	1.3		
7.5	10	6.8	2.5	0.9	0.2		
5	10	3.3	1.3	0.4	0		
2.5	9	3.3	1.7	0.4			
1	9	0.3	-0.1	-0.2			

It will be seen from Fig. 1 that the highest recorded effect was obtained 15 minutes after dosing. This was the case for other drugs examined in the same way.

In order to use this method for assay, the relationship between dose and response must be determined, and to do this it is necessary to decide on a measure of the response to any given dose. For this we could take either (a) the effect at a given time after dosing, or (b) the time taken to produce a given effect. The former is the easier to determine and the more reliable. The recorded effect at 15 or at 30 minutes after dosing or a value obtained by smoothing the curve might be used, but there is little to be gained by the latter procedure since by far the

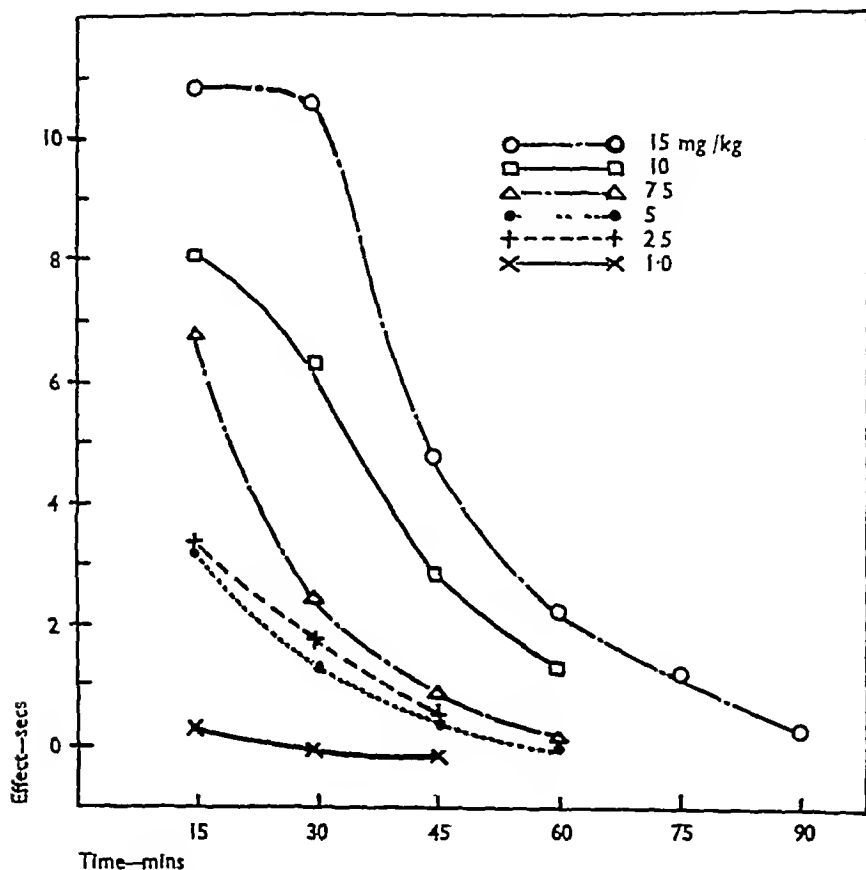


FIG 1—Analgesic action of pethidine. Effect plotted against time for doses from 1 to 15 mg./kg

greatest source of error is the variation between the rats themselves. From an examination of the results for pethidine and other drugs the recorded effect at 15 minutes was found to be a better measure than that at later times because a smoother and steeper curve was thereby obtained for the relationship between dose and response. It was therefore adopted as the basis of assay.

The reaction time at any given time after dosing for each rat is expressed as an average of three readings. The variation between repeat readings is considerably less than the variation between individual rats and therefore no worthwhile gain in accuracy can be obtained by taking more than three. A further appreciable increase in accuracy could only be obtained by increasing the number of rats used.

The mean effects 15 minutes after injection, the doses, and the number of rats per dose for a number of drugs are given in Table II. In Figs 2 *et seq* the mean effect at 15 minutes is plotted against log dose.

TABLE II

Drug	Test									
Morphine	A	Dose mg/kg	10	5	4	3	2.5	2	1.5	1
		No rats	3	3	9	10	10	10	10	11
		Mean effect (secs)	10.5	10.8	9.5	8.2	4.7	3.6	1.9	0.2
Codeine	A*	Dose mg/kg	25	20	15	12.5	10	7.5	5	
		No rats	4	5	9	10	10	—	4	
		Mean effect (secs)	10	9.9	10.1	7.8	1.1	—	0.4	
	B	No rats	—	—	10	10	10	10	10	
		Mean effect (secs)	—	—	8.7	5	5.1	3.2	1.1	
Pethidine	A	Dose mg/kg	15	10	7.5	5	2.5	1		
		No rats	5	9	10	10	9	9		
		Mean effect (secs)	10.8	8.1	6.8	3.3	3.3	0.3		
Hashish distillate	A	Dose mg./kg	3	2	1	0.75	0.5	0.25	0.1	
		No rats	10	10	10	10	10	10	10	
		Mean effect (secs)	9.1	8.1	6.5	4	1.7	3.5	0.8	
Phenazone	A	Dose mg/kg	500	350	250	150	100			
		No rats	9	10	15	15	10			
		Mean effect (secs)	9.8	8.3	6.2	3.8	1.8			
Aspirin	A	Dose mg/kg	725	600	500	425	350			
		No rats	10	9	10	10	10			
		Mean effect (secs)	7.4	6.4	5.4	3.9	2.7			
	B	No rats	8	9	10	10	10			
		Mean effect (secs)	8.8	6.3	4.1	4.1	2.9			
Pheno-barbitone	A	Dose mg./kg	100	75	50	37.5	25	17.5	10	
		No rats	26	28	32	30	35	30	29	
		Mean effect (secs)	0.4	1.3	1.8	2.1	3.9	1.8	0.5	

\* The figures for Test A were obtained in preliminary experiments before the apparatus and technique were standardized.

The relationship is substantially linear over a wide range for all the drugs examined with the exception of phenobarbitone, which will be discussed below. For extreme maximal and minimal effects the curve flattens out as, of course, it must. No transformation of the effect variable is thus required provided reasonable care is exercised with extreme values. In certain cases it is well to omit

results obtained with doses at the extreme ends of the range—for example, of the figures given in Table II, that obtained with 10 mg/kg morphine should be ignored. In an assay of the activity of a sample of a known drug, a range of doses which produces intermediate effects, e.g., 2 to 8 seconds, should be used.

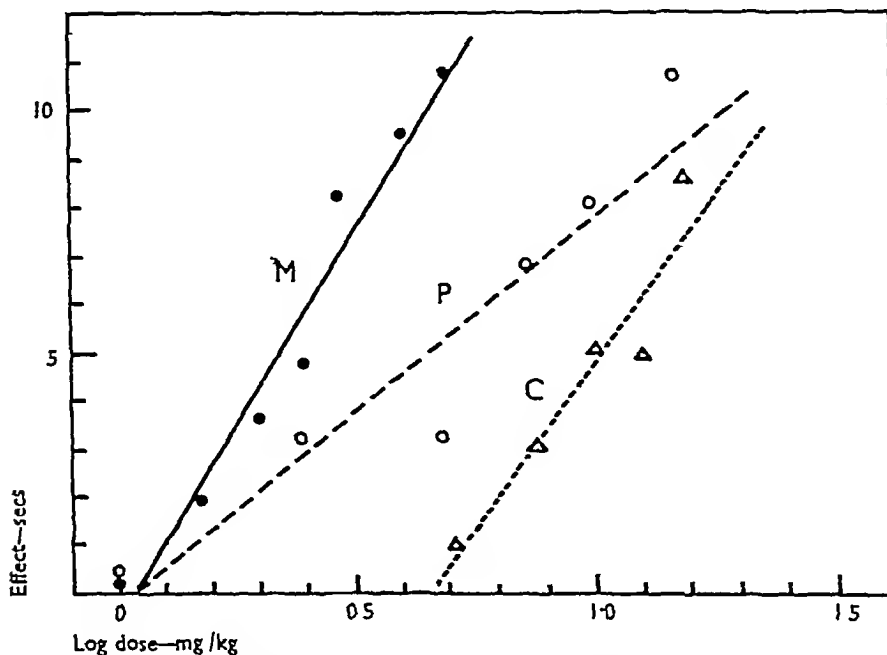


FIG 2—Analgesic action of morphine (M), codeine (C), and pethidine (P) administered intravenously. Effect at 15 minutes after injection plotted against log. dose

### Sources of error

The main source of error in determining the log dose-response line is the variation between individual rats. In our experiments, this variation was related to the magnitude of the mean effect, being least for maximal and minimal effects. Except for these extreme values, however, the standard deviation was practically constant for different doses. Its average value was about 3 seconds for all the drugs examined.

Another source of error is that different results may be expected from experiments carried out on different days. When comparing the activity of two or more samples of any drug, the variation due to this factor may be eliminated by ensuring that in each group tested at one time all samples are equally represented. There is evidence that error has arisen from this source in the results reported here, but owing to the accidental arrangement of the treatments it has not been serious.

An overall measure of the error in these experiments may be obtained by calculating the variance about the best line of fit. Combining the results of all the experiments except those with phenobarbitone, we find that the variation about the linear regressions is no greater than expected from the standard deviation of 3 seconds for individual rats in each group. This indicates that the linear regressions give a satisfactory fit.

From the regressions we may calculate the doses required to produce an increase in reaction time of 5 seconds. These are shown in Table III together with the slopes of the regression lines for the various drugs examined (except phenobarbitone)

TABLE III

Drug	Slope	Standard error of slope	Dose to give mean response of 5 seconds	Standard error	Expected standard error of comparison of two samples with 100 rats each
Morphine	16.1	1.8	mg./kg. 2.2	% 6	% 6.5
Codeine (Test B)	14	2.5	10	7.5	7.5
Pethidine	8.1	1.1	4.6	13	13
Hashish	5.7	0.8	0.72	16	19
Aspirin (Test A)	15	3.8	490	7	7
Aspirin (Test B)	17.4	4	490	7	
Phenazone	11.6	1.7	169	8.5	9

The slopes for morphine, codeine, aspirin, and phenazone do not differ significantly from one another. The slopes for pethidine and hashish are significantly lower than the remainder with the possible exception of phenazone.

The standard errors quoted above refer to the reproducibility within the conditions of the experiment. It is to be expected that larger variations would arise, especially in the dose required to give a mean response of 5 seconds, if a different batch of animals were used or if the experiment were repeated at a different time. Some measure of control is obtained by selecting rats with normal reaction times of 4 to 6 seconds, but we cannot say how effective this is in controlling the response at any given dose level.

The figure in the last column in Table III represents the standard error of a comparison of the activity of two samples of each drug when 100 rats are used for each sample and the following conditions are observed:

(a) That the two samples are equally represented in each group of rats treated and tested together.

(b) That the order in which the doses are given is randomized

(c) That the dose range is so chosen that the mean effect in all cases falls on the linear portion of the log dose-response curve (Simplification of the analysis results if the doses are taken at equal logarithmic intervals)

If the experiment continues over two days the whole test should be considered as two complete replicates, with 50 rats per sample in each. This idea can be carried further and the experiments regarded as 4 complete replicates with 25 rats per sample in each. Further subdivision is impracticable.

For comparing the analgesic potency of different drugs we adopt as a standard of comparison the dose of each required to produce an increase in mean reaction time of 5 seconds. This increase represents approximately 50 per cent of the maximum increase which can be measured.

The doses of various analgesics required to produce this effect are shown in Table IV.

TABLE IV

Drug	Dose required to produce an increase in mean reaction time of 5 seconds	
	Intravenous administration	Intraperitoneal administration
	mg./kg.	mg./kg.
Morphine	2.2	10
Codeine	10	30
Pethidine	4.6	30
Aspirin	490	Inactive
Phenazone	169	"
Hashish distillate	0.72	"
Phenobarbitone	*25	"

\* This dose produced an increase of only 4 seconds, the maximal obtainable with phenobarbitone.

Further observations upon the analgesic action of these drugs are given below.

1 *Morphine and codeine*.—Both these are active when given by intraperitoneal or intravenous injection. Rats dosed with them and showing pronounced analgesia as indicated by a marked increase in reaction time appeared normal in other respects. From Table IV it appears that morphine is about five times as active as codeine when both are given intravenously and three times as active intraperitoneally. This is in fair agreement with clinical experience and with the results obtained by Woolfe and MacDonald (1944), using mice.

2 *Pethidine*.—The response produced by increasing intravenous doses of pethidine is shown in Figs 1 and 2. The slope of the log dose-response curve is somewhat less than that for morphine or codeine, indicating that with increasing doses of this drug its analgesic action, relative to that of either of the others, becomes less.

This result confirms that of Woolfe and MacDonald (*loc cit*) and suggests that pethidine is relatively less active than morphine in the control of the more intense forms of pain

3 *Aspirin and phenazone*—As already mentioned, aspirin produces detectable effects after intravenous administration only. Even so the dose required to produce any effect is enormously greater than with pethidine or the opiates

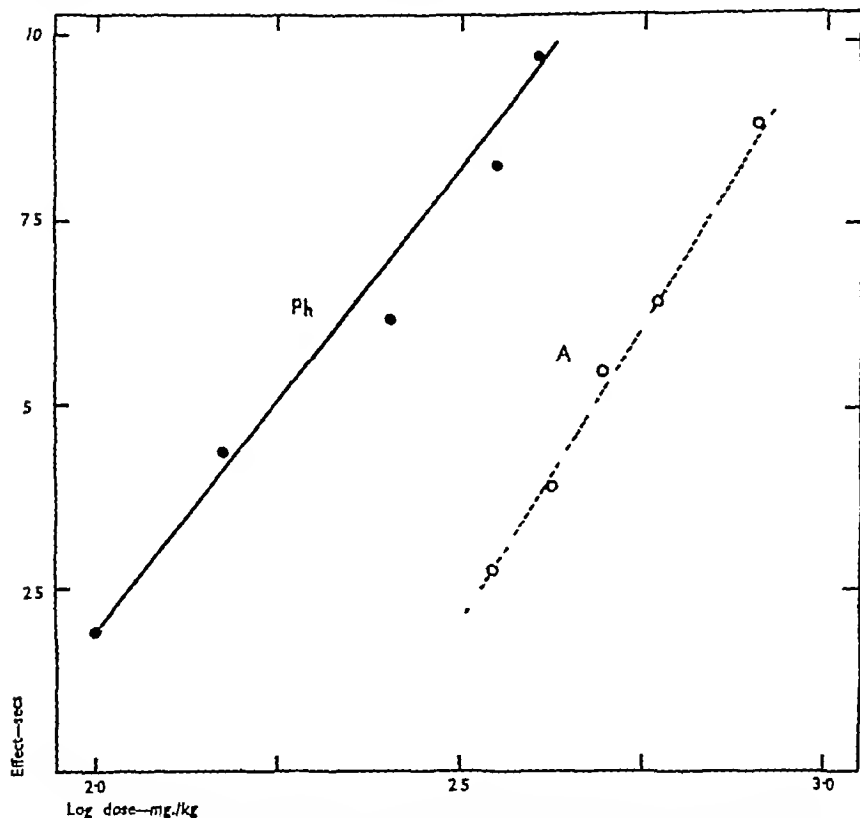


FIG 3—Analgesic effect of phenazone (Ph) and aspirin (A), administered intravenously. Effect at 15 minutes after injection plotted against log dose

Phenazone has a slight action when given intraperitoneally in large doses just below the median lethal. When given intravenously, it produces clearly demonstrable effects at lower doses (Fig 3)

4 *Hashish*—A sample of total distillate of hashish, for which we are indebted to Professor A R Todd, was examined by our technique. No analgesic effect was observed when a fine aqueous emulsion of the distillate was given intraperitoneally

or intravenously to rats. With the highest doses given the animals died in convulsions shortly after being dosed.

Acetone solutions of the distillate, given intravenously, produced analgesic effects, but the results were vitiated by damage to the tails at the site of injection and the animals developed haematuria. After trials with various solvents we found that by diluting an acetone solution of the distillate with defibrinated rat blood a preparation suitable for assay by our technique was obtained. Great care had to be taken to keep the acetone content to a minimum, otherwise intense haematuria resulted. Not more than 0.1 ml of acetone solution to every 1 ml of blood was finally used in our experiments. Used in this way the distillate produced measurable effects in very small doses (0.1 mg/kg). "Complete" analgesia was

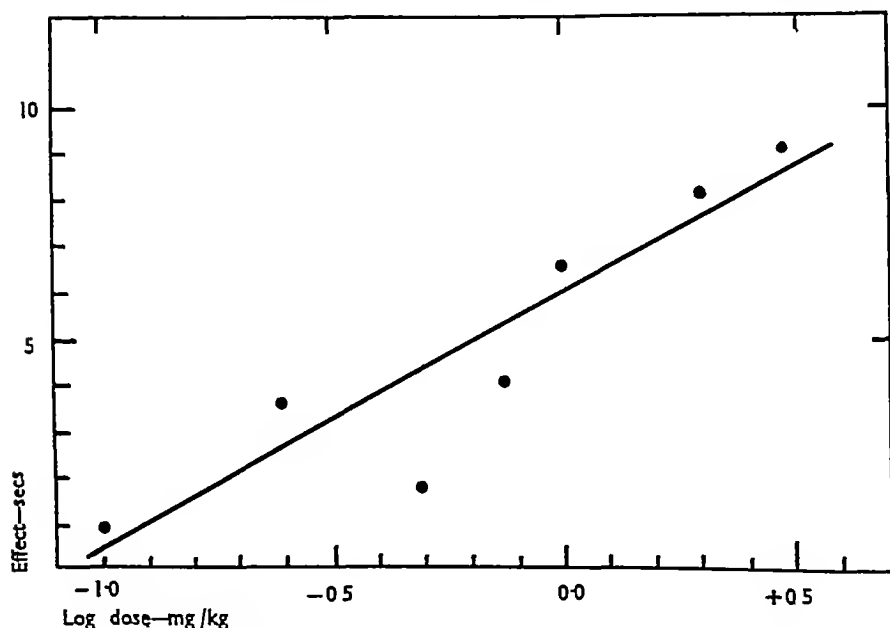


FIG. 4—Analgesic action of hashish distillate administered intravenously. Effect at 15 minutes after injection plotted against log. dose.

only obtained with a dose fifty times as great as this, i.e., 5 mg/kg, which is very close to the median lethal. The slope of the log dose-response curve (Fig. 4) was the lowest obtained with any drug examined. This is probably due to the fact that the sample tested was a mixture of the several active constituents of hashish.

**5 Phenobarbitone**—Barbiturates are reputed to be mild analgesics. We were unable to demonstrate any analgesic effect with phenobarbitone given intraperitoneally in doses up to and including hypnotic doses. With still higher doses, near to the median lethal, an effect is obtained which we attribute to the general depressant action of the drug. On the other hand if phenobarbitone is given intravenously analgesia is produced by doses between 10 to 100 mg/kg. As the



dose is increased from 10 mg/kg the analgesic effect increases to a maximum which is obtained with a dose of about 25 mg/kg. Further increase in the dose leads to a diminution of the effect until when hypnotic doses are attained analgesia

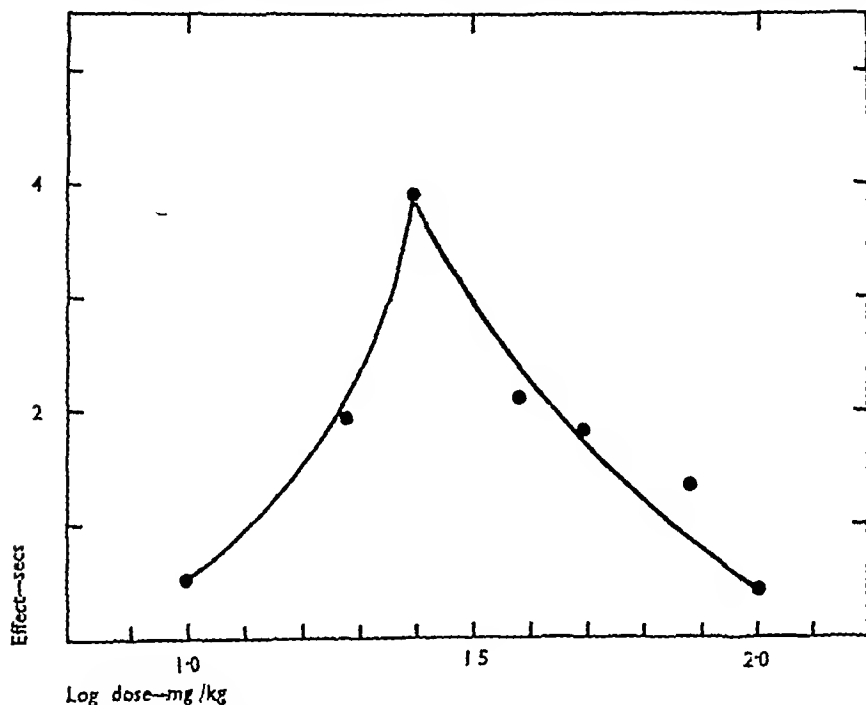


FIG 5—Analgesic effect of phenobarbitone administered intravenously. Effect at 15 minutes after injection plotted against log dose

is no longer observed. The results reproduced in Table II and Fig 5 are the means of three different experiments using about 70 rats in each.

It is of interest to note that the analgesic effect of morphine can be demonstrated in rats fully anaesthetized with phenobarbitone. The results demonstrate the complexity of the action of analgesic and hypnotic drugs.

#### SUMMARY

A new method is described for the detection and evaluation of analgesic activity, making use of the response of rats to a heat stimulus applied to the tail.

By the use of this method the analgesic activity of morphine, codeine, pethidine, aspirin, phenazone, hashish, and phenobarbitone have been compared.

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# THE PHARMACOLOGICAL PROPERTIES OF $\alpha$ $\beta$ DIHYDROXY-*r*-(2-METHYLPHENOXY)- PROPANE (MYANESIN)

BY

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During a systematic investigation of the pharmacological properties of  $\alpha$ -substituted ethers of glycerol the observation was made that certain of these compounds produced paralysis with profound muscular relaxation. Administration of small quantities of these substances to mice, rats, or guinea pigs caused tranquillization, muscular relaxation, and a sleep-like condition from which the animals could be roused. Larger doses produced ataxia, which was followed by paralysis. The animals did not react to painful stimuli and were unable to turn over when placed on their backs, all muscles were well relaxed and quite limp. Paralysis was followed by complete recovery. Excitement, tremors, twitchings, or convulsions did not occur at any time after administration of the drug.

The relative toxicity and effectiveness of the most active compounds after subcutaneous administration to white mice are shown in Table I, together with their melting points and solubilities in water.

Suspensions prepared by mixing one volume of a 40 per cent w/v alcoholic solution of the drug with 7 volumes of an aqueous 5 per cent w/v gum acacia solution were used because of the low water solubility of several of the drugs. The inability of an animal to turn over when placed on its back, together with the absence of any movements of the limbs, was taken as the criterion of paralysis. The animals were observed for 14 days after administration of the drug but any deaths usually occurred within 24 hours of the administration. The observed percentages of effect were treated according to Dragstedt and Langs (1928) method and the probits of these deduced percentages were plotted against the logarithms of the doses. The median paralyzing and lethal doses were then found graphically and the standard errors estimated according to the method of Miller and Tainter (1944). The therapeutic index was expressed by the ratio

$$\frac{\text{median lethal dose}}{\text{median paralyzing dose.}}$$

Most of the 143 compounds which were examined produced paralysis only in doses which were lethal to a proportion of the animals. No correlation of paralyzing activity with chemical constitution was observed.  $\alpha$   $\beta$ -dihydroxy-*r*-

(2-methylphenoxy)-propane, the *o* tolyl ether of glycerol, was the most potent and safest of all the compounds examined and had the widest margin between the paralyzing and lethal doses. This substance has been named 'myanesin' In

TABLE I  
MEDIAN LETHAL AND PARALYSING DOSES OF  $\alpha$ -SUBSTITUTED ETHERS OF GLYCEROL  
IN WHITE MICE AFTER SUBCUTANEOUS ADMINISTRATION

R-O-CH <sub>2</sub> -CHOH-CH <sub>2</sub> OH R-substituent	Melting point	Water solubility % w/v at room temp	No of mice	LD50 $\pm$ S.E. mg. per kg	PD50 $\pm$ S.E. mg. per kg.	Therapeutic index LD50/PD50
<i>n</i> -Butyl	liquid	1.0	60	2,800 $\pm$ 150	1,480 $\pm$ 54	1.89
<i>n</i> -Amyl	liquid	0.1	80	2,000 $\pm$ 100	870 $\pm$ 49	2.30
<i>Iso</i> -Amyl	liquid	0.1	50	2,100 $\pm$ 130	1,240 $\pm$ 80	1.69
<i>n</i> -Hexyl	liquid	0.05	90	2,230 $\pm$ 50	1,060 $\pm$ 70	2.15
Phenyl	55-57° C	0.7	90	1,680 $\pm$ 65	920 $\pm$ 98	1.82
<i>p</i> -Chlorophenyl	77-79° C	0.08	120	920 $\pm$ 86	420 $\pm$ 46	2.19
2,4-Dichlorophenyl	74-76° C	0.001	60	840 $\pm$ 44	540 $\pm$ 53	1.55
<i>p</i> -Bromophenyl	74-75° C	0.03	50	1,160 $\pm$ 57	840 $\pm$ 44	1.38
<i>o</i> -Tolyl (Myanesin)	70-71° C	1.09	120	1,000 $\pm$ 56	325 $\pm$ 20	3.07
<i>m</i> -Tolyl	63-66° C	0.5	80	1,470 $\pm$ 89	570 $\pm$ 51	2.58
<i>p</i> -Tolyl	74-75° C	0.2	90	1,270 $\pm$ 61	530 $\pm$ 39	2.39
<i>p</i> -Ethylphenyl	67-69° C	0.08	50	1,450 $\pm$ 67	820 $\pm$ 38	1.77
<i>p</i> -Methoxyphenyl	80-81° C	0.8	70	1,610 $\pm$ 50	940 $\pm$ 74	1.72

the following paper a description of its pharmacological properties is given. A preliminary note on its pharmacology (Berger and Bradley, 1946) and a report on its clinical action have been published.

*Chemical and physical properties of myanesin*—Myanesin is a colourless, odourless, crystalline solid, melting point 70-71° C. Solubility at 22° C. is 1.09 g per 100 ml water, but relatively stable supersaturated solutions can easily be obtained by cooling solutions prepared at higher temperatures. It is very soluble in ethyl alcohol and propylene glycol. Urea and its derivatives, particularly ethyl urea, greatly increase the water solubility of myanesin. Solutions of the drug are stable, can be sterilized by heating, and are compatible and freely miscible with solutions of sodium chloride, glucose, and derivatives of barbituric and thiobarbituric acid.

*Toxic and paralyzing doses in mice*—The median lethal and median paralyzing doses after intravenous administration of aqueous solutions to mice were 322  $\pm$  11 mg per kg and 150  $\pm$  6 mg per kg respectively. The injections were spread over a period of 1 minute. The results obtained after intraperitoneal administration to mice are given in Table II.

*Effect on rabbits*—Rabbits injected intravenously with 10 to 12 mg per kg showed the head drop sign which is used in the standardization of certain curare preparations (Bennett, 1941). Intravenous injection of 50 mg per kg caused complete paralysis about 10 seconds after termination of the injection. The animal,

although unable to move, gave the impression of being conscious. Skeletal muscles were flaccid. The corneal reflex was present, but the retraction reflex was absent. Breathing was regular, mainly diaphragmatic and somewhat

TABLE II

INCIDENCE OF PARALYSIS AND DEATH, AND DURATION OF INDUCTION AND PARALYSIS IN MICE AFTER INTRAPERITONEAL ADMINISTRATION OF AQUEOUS SOLUTIONS OF MYANESIN. TWENTY MICE WERE USED AT EACH DOSE LEVEL.

Dose mg/kg	Paralysed per cent	Died per cent	Mean duration of induction min and sec.	Mean duration of paralysis min and sec.
150	0	0	—	—
175	65	0	2 ± 6"	12 ± 142"
200	70	0	2 ± 6"	13 ± 418"
225	93	0	1 48" ± 12"	23 ± 412"
300	100	0	1 36" ± 12"	25 ± 248"
350	100	0	1 12" ± 6"	56 ± 642"
500	100	5	0'48" ± 2"	61' ± 12'24"
550	100	35	—	—
600	100	45	0'48" ± 6"	120 ± 418"
650	100	60	—	—

LD50, 610 ± 101 mg./kg. PD50, 178 ± 88 mg./kg. Therapeutic index, 3.42

increased in rate. The posterior half of the animal remained paralysed for a longer period of time than the anterior half. The rabbits regained muscular power about 7 minutes after termination of the injection and did not show any untoward symptoms, either immediate or delayed. The injection of larger doses caused paralysis of longer duration. The largest tolerated dose on rapid intravenous injection was about 100 mg per kg. Doses of 200 mg per kg injected at a rate of 100 mg per minute, and 350 mg per kg injected over a period of 30 minutes, were tolerated. The animals recovered about 20 minutes after termination of the injections and remained well.

*Absorption and fate*—The drug was well absorbed from the blood, muscle, subcutaneous tissues, peritoneal cavity, stomach, and rectum and could be effectively administered by any of these routes. Clearance rates were investigated in rabbits and cats. A 1.8 per cent aqueous solution of myanesin was injected at a constant rate into the marginal ear vein of non-anaesthetized rabbits for several hours. Cats were anaesthetized with ether and prepared for recording blood pressure and respiration; the myanesin solution was infused into the femoral vein. As soon as the infusion was started, no further ether was given, as the procedure caused paralysis with complete relaxation. It was found that both rabbits and cats tolerated the infusion of at least 4.5 mg of myanesin per min per kg body weight for 3 or 4 hours. This procedure caused paralysis with complete relaxation. Apparently myanesin was quickly metabolized or changed to a physiologically inactive compound in the body. The urine collected from rabbits after large doses of the drug did not cause paralysis in mice.

The drug did not possess cumulative action and tolerance to it did not occur. A minimum effective dose given daily to mice produced the same effect on 14 successive administrations

Toxic doses caused death by respiratory failure. The heart, as a rule, continued beating after the respiration had ceased. Even after rapid intravenous injection of toxic doses struggling and convulsions were not observed

*Chronic toxicity*—Young growing rats were fed for 9 weeks on a diet containing 2 per cent of myanesin. Each rat consumed on the average 0.18 g of the drug per day. All the animals survived and continued to grow, although they did not gain weight as rapidly as the controls, which were litter mates. This was due to the fact that the experimental animals consumed less food than the controls, possibly because of the unpalatability of the drug-containing diet.

The animals were sacrificed 63 days after the beginning of the experiment. Six (30 per cent) out of 20 treated rats showed calculi in the bladder and also had small abscesses in the submaxillary glands. The kidneys, liver, spleen, lungs, heart, and suprarenal glands did not show any macroscopical or microscopical lesions.

*The effect on blood pressure and respiration*—Experiments were carried out on rabbits and cats under ether and hexobarbitone anaesthesia. An intravenous injection of 30 mg myanesin did not cause any alterations in blood pressure or respiration. Fairly rapid intravenous administration of 50 mg caused a slight or moderate fall of blood pressure amounting to 25 to 40 mm Hg. There was also a decrease in rate and an increase in depth of the respiratory movements. Both respiration and blood pressure returned to normal after 4 to 8 minutes. Large intravenous doses of the order of 125 mg caused large falls of blood pressure and cessation of respiratory movements for 10 or 15 seconds, but respiration restarted spontaneously and the animals recovered. The blood pressure depression may have been caused partly by accommodation of more blood in the muscles owing to muscular flaccidity. Doses of 150–200 mg intravenously caused respiratory arrest and death.

*Comparison with curare*—In frogs flaccid paralysis and cessation of respiratory movements were obtained after intralymphatic injections of 3 to 10 mg per frog (*R. temporaria*). The frogs remained paralysed for several hours, but recovered completely by the next day. In paralysed animals faradization of the sciatic nerve failed to produce muscular contractions, whereas direct stimulation of the muscle itself always produced contractions. The threshold for direct stimulation during the pre-paralytic and paralytic stage remained unchanged. The results indicate that the drug may possess a curare-like action when administered in large doses. The effect of myanesin on mammalian nerve-muscle preparations was similar to that of curare, but large and nearly lethal doses were required for its production.

The paralysis of the muscle to indirect but not to direct stimulation, which was observed only when large doses of myanesin were given, may be due either to a block at the myoneural junction (curare-like action) or to direct action on the nerve (local anaesthetic action). Isolated nerve-muscle preparations could not be usefully employed for the demonstration of curare-like action because myanesin on local application impaired the conductivity of the nerve.

**Physostigmine antagonism**—The effect of physostigmine sulphate and of prostigmin on the action of myanesin was investigated in frogs and mice. Physostigmine (0.02 mg per frog) somewhat accelerated recovery from paralysis caused by the injection of 3 mg myanesin (mean recovery time of 9 control frogs  $326 \pm 29$  min, mean recovery time of 9 physostigmine frogs  $237 \pm 27$  min,  $t=2.23$ ,  $P<0.05>0.02$ ). Physostigmine also caused resumption of respiratory movements and the appearance of generalized muscular fibrillation.

In mice subcutaneous injections of prostigmin (0.15 mg per kg) somewhat accelerated recovery from paralysis caused by myanesin. Larger but tolerated doses of prostigmin when injected together with non-lethal doses of myanesin caused death in the majority of animals. Neither prostigmin nor physostigmine abolished the effect of lethal doses of myanesin. This suggested that the curare-like activity of myanesin accounted for only a part of the effects produced by this drug.

**The anticonvulsive action**—The depressant action of myanesin on the central nervous system was demonstrated and located by its power to antagonize convulsions produced by central nervous system stimulants. Convulsions were produced by leptazol or strychnine and antagonized by simultaneous administration of myanesin or soluble hexobarbitone. All solutions contained  $2\frac{1}{2}$  per cent w/v gum acacia and were injected subcutaneously in a volume of 0.4 ml per 20 g mouse.

TABLE III

THE ANTAGONISM OF MYANESIN AND SOLUBLE HEXOBARBITONE TO LEPTAZOL AND STRYCHNINE CONVULSIONS ON SUBCUTANEOUS ADMINISTRATION TO MICE

Leptazol mg./kg.	Strychnine mg/kg	Myanesin mg /kg.	Hexobarbitone mg./kg	No of mice	Per cent convulsed	Per cent died
120	—	—	—	60	96.6	53.3
120	—	500	—	20	15.0	0.0
120	—	400	—	30	46.6	3.3
120	—	200	—	40	92.5	42.5
120	—	—	50	20	0.0	0.0
—	133	—	—	109	68.9	49.6
—	133	100	—	140	19.3	2.8
—	—	50	—	50	40.0	12.0
—	133	—	100	100	97.0	29.0
—	133	—	50	20	95.0	35.0

Large doses of myanesin diminished the incidence and severity of leptazol convulsions (Table III). Mice receiving 500 mg per kg myanesin survived and usually did not convulse, 400 mg per kg markedly reduced mortality but did not prevent the occurrence of convulsions, smaller doses were almost ineffective.

antagonistic effect to leptazol shows that the midbrain is influenced only when large doses are given. Myanesin does not appear to act on the brain because it does not affect consciousness and never causes prenarcoctic excitation. It cannot, therefore, be classed among the general anaesthetics.

Animals which have been completely paralysed by myanesin continue to breathe spontaneously and recover from the effects of the drug without the aid of artificial respiration. Lethal doses of myanesin eventually paralyse the diaphragm also. It may be concluded that the diaphragm is less affected by myanesin than other muscles and that it is the last muscle to be paralysed. In this respect myanesin also differs from curare. With curare complete muscular paralysis without arrest of respiration can hardly be produced and curarization of the respiratory muscles does not seem to be markedly less complete than that of the other skeletal muscles (Cohnberg, 1946).

In preliminary clinical trials myanesin has proved useful for the production of muscular relaxation during light anaesthesia. It may also be of value in cases of spastic paralysis and for the prevention of traumatic accidents during convulsive shock therapy.

#### SUMMARY

1 A number of  $\alpha$ -substituted ethers of glycerol produced transient relaxation and paralysis of skeletal muscles in small laboratory animals.

2 Of the 143 compounds which have been examined,  $\alpha$   $\beta$ -dihydroxy- $\gamma$ -(2-methylphenoxy)-propane, named myanesin, was the most potent and also possessed the widest margin of safety between paralysing and lethal doses.

3 Myanesin in non-paralysing doses effectively antagonized strychnine convulsions but was not very effective against leptazol convulsions. Myanesin also counteracted prenarcoctic excitement and increased the duration of barbiturate anaesthesia.

4 Myanesin in small doses produced depression of the reflex excitability of the spinal cord. Larger doses had an ascending depressant action on the central nervous system. Myanesin also possessed local anaesthetic properties.

5 Myanesin may prove useful for the production of muscular relaxation during light anaesthesia.

#### ACKNOWLEDGMENTS

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# THE SYNTHESIS, TOXICITY AND ANAESTHETIC POTENCY OF TWO NEW LOCAL ANAESTHETICS

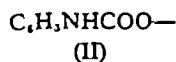
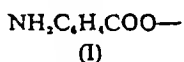
BY

YAO-TSENG HUANG, MING-CHENG LU, AND I CHANG

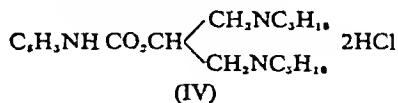
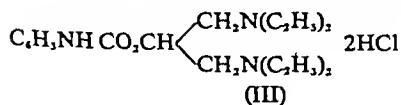
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Einhorn in 1899 demonstrated the production of local anaesthesia by esters of *p*-aminobenzoic acid and in 1905 introduced *p*-aminobenzoyldiethylamino-ethanol (procaine) as a local anaesthetic. Since then an enormous number of compounds chemically related to procaine have been prepared and many of them have proved useful for the production of local anaesthesia in one or another of several fields. On the other hand, comparatively few studies on the local anaesthetic properties of esters of phenylcarbamic acid (II) have been made, although chemically they are closely related to esters of *p*-aminobenzoic acid (I) as shown below



The object of the present paper is to report our studies upon the synthesis, toxicity, and local anaesthetic activity of two new esters of phenylcarbamic acid, viz., sym bis-(diethylamino)isopropyl phenylurethane dihydrochloride (III) and sym dipiperidino-isopropyl phenylurethane dihydrochloride (IV)



## EXPERIMENTAL

*Sym bis-(diethylamino)isopropylalcohol*—A mixture of sym. dichloroisopropylalcohol (10 g) and diethylamine (22.6 g) was refluxed for sixteen hours. It was then acidified with dilute hydrochloric acid (30 ml) and washed with ether so as to remove any unchanged sym. dichloroisopropylalcohol. The addition of solid sodium hydroxide (15 g.) to the aqueous solution precipitated an oil which was taken up in ether. The ethereal extract was dried over potassium carbonate and then distilled to remove the solvent and excess diethylamine. The residue upon further distillation gave 11.0 g (or 70% of the theoretical yield) of pure sym. bis-(diethylamino)isopropyl alcohol, b.p. 93/62 mm or 230–232/769 mm. Berend (1884) obtained the same compound by a different process and gave its boiling point as 234.5°.

*Sym bis-(diethylamino)isopropyl phenylurethane dihydrochloride (III)*—Sym. bis-(diethylamino)isopropylalcohol (4 g) previously cooled to 0° was mixed thoroughly with phenyl-isocyanate (2.63 g.) and allowed to stand at 0° for one hour and then at room



temperature overnight. Since the phenylurethane thus formed failed to solidify upon cooling to  $-12^{\circ}$ , it was treated with 40 ml. 10% hydrochloric acid. The resulting solution, after being freed from oily impurities by means of animal charcoal and dried *in vacuo* over sulphuric acid and solid sodium hydroxide, gave 71 g of almost pure sym bis-(diethylamino)isopropyl phenylurethane dihydrochloride, mp 205–210. It crystallized from absolute alcohol in colourless crystals, extremely soluble in water, mp 210–211 (bath preheated to  $200^{\circ}$ ).

Found C, 54.93, H, 8.42, N, 10.48  $C_{11}H_{21}O_2N_2Cl$ , requires C, 54.80, H, 8.44, N, 10.66%

*Sym dipiperidino-isopropylalcohol*—Sym. dichloro-isopropylalcohol (6 g.) and piperidine (16 g.) were mixed and allowed to stand at  $0^{\circ}$  for one hour and then at room temperature for twenty hours. The mixture was then heated at  $60-70^{\circ}$  for six hours and finally on a boiling water bath for five minutes. The mixture was diluted with water (20 ml.) and extracted several times with ether. The ethereal extract was dried over potassium carbonate, evaporated on the water bath and filtered to remove solid impurities. Pure sym. dipiperidino-isopropylalcohol distilled at  $144-145/6.5$  mm., yield, 6.9 g., or 66% of the theoretical. Vassiliades (1937) gave  $172-173^{\circ}/12$  mm. as the boiling point of the compound.

Found C, 69.29, H, 11.39. Calcd for  $C_{11}H_{21}ON_2$ , C, 68.96, H, 11.58%

The 3,5-dinitrobenzoate was prepared, which crystallized from petroleum ether (bp  $30-50^{\circ}$ ) in yellowish plates, mp  $98-100^{\circ}$ .

Found C, 57.21, H, 6.73. Calcd for  $C_{21}H_{23}O_5N_4$ , C, 57.11, H, 6.71%

*Sym dipiperidino-isopropyl phenylurethane*—Sym. dipiperidino-isopropylalcohol (4 g.) and phenylisocyanate (2.72 g.) were mixed at  $0^{\circ}$ , the reaction took place immediately with evolution of heat. After being kept in ice for a half-hour the mixture was allowed to stand at room temperature for sixteen hours. The resulting product, sym. dipiperidino-isopropyl phenylurethane, was extracted with petroleum ether (bp  $40-60^{\circ}$ ) in a Soxhlet apparatus. It crystallized in colourless prisms, melting at  $106-107^{\circ}$ . Yield, 5.3 g., or 87% of the theory.

Found C, 69.60, H, 8.87, N, 11.91  $C_{21}H_{23}O_2N_4$ , requires C, 69.51, H, 9.05, N, 12.17%

*Sym dipiperidino-isopropyl phenylurethane dihydrochloride* (IV).—The hydrochloride was prepared by dissolving the phenylurethane (1.5 g.) in ice-cold 5% hydrochloric acid (30 ml.) and evaporating the solution *in vacuo* over sulphuric acid and solid sodium hydroxide. The hydrochloride melted at  $242-243^{\circ}$  (bath previously heated to  $230^{\circ}$ ) and crystallized from warm dilute hydrochloric acid in colourless needles, readily soluble in water. The yield was quantitative.

Found C, 57.15, H, 7.95, N, 9.75  $C_{21}H_{23}O_2N_4Cl$ , requires C, 57.39, H, 7.95, N, 10.05%

### TOXICITY

The determination of the LD50 of sym. dipiperidino-isopropyl phenylurethane dihydrochloride (hereafter referred to as PP) and sym. bis-(diethylamino)-isopropyl phenylurethane dihydrochloride (hereafter referred to as DP) was carried out on mice according to Kärber's method. For the purpose of comparison, the LD50s of cocaine and procaine were also determined. All drugs were given intraperitoneally. Table I shows that PP and DP are about as toxic as cocaine but several times more toxic than procaine.

TABLE I  
THE LD<sub>50</sub> OF PP, DP, COCAINE, AND PROCAINE

Drug	Dose, mg./kg	Proportion killed	LD <sub>50</sub> , mg./kg.
PP	50	0/10	108
	70	2/10	
	90	3/10	
	110	7/10	
	130	10/10	
DP	90	0/10	151
	110	1/10	
	130	3/10	
	150	7/10	
	170	10/10	
Cocaine	60	0/12	91
	70	1/12	
	80	4/12	
	90	6/12	
	100	12/12	
Procaine	300	0/10	564
	400	2/10	
	500	5/10	
	600	7/10	
	700	10/10	

In order to compare the toxic action of the above drugs upon respiration and circulation, experiments were made on etherized cats, and the doses of the drugs which caused an appreciable depression of blood pressure and respiration were determined. The drugs were injected intravenously. The average results obtained on six cats are shown in Table II. These results show that PP and DP have an action on blood pressure and respiration more or less similar to that of cocaine but stronger than that of procaine.

TABLE II  
DOSAGE OF PP, DP, COCAINE, AND PROCAINE REQUIRED TO PRODUCE AN APPRECIABLE DEPRESSION OF BLOOD PRESSURE AND RESPIRATION

Drug	Dose, mg./kg.	
	Circulation	Respiration
PP	1.0	1.8
DP	1.1	2.2
Cocaine	1.0	1.7
Procaine	6.0	7.0

#### ANAESTHETIC POTENCY

The anaesthetic potencies of PP and DP were compared with those of cocaine and procaine by the rabbit's cornea method and the intradermal weal method.

These methods were chosen because they resemble more closely the clinical methods of applying local anaesthetics and also because they appear to give more reliable results than other methods (Sinha, 1936)

*Rabbit's cornea method*—In this method the cornea was flooded with the drug solution and washed thoroughly with saline solution at the end of five minutes. The winking reflex was tested from time to time until it was completely restored to normal. Since there was marked individual variation in the sensitivity of the rabbit's eye toward anaesthetics and in some animals the sensitivity varied from time to time, comparisons with cocaine were made by applying the drug to be tested alternately to the same eye of a rabbit whose sensitivity showed little or no variation with time. In order to provide time for the recovery of the eyes, an interval of six to seven days was allowed between tests. Rider (1930) and Sinha (1936) have both asserted that repeated applications of cocaine render a cornea less sensitive to the drug. On the other hand, the writers found more often a slight increase rather than a decrease in the sensitivity of the rabbit's eye when cocaine was applied to it every six to seven days. Table III shows the anaesthetic potency of PP and DP and cocaine as determined by the rabbit's cornea method. The results are the average of two or three more or less consistent determinations, discordant results being discarded. It will be seen from Table III that while PP is about twice as potent as cocaine, DP is only one half as potent. Since the toxicity of these agents is about equal to that of cocaine, these results would warrant a clinical trial of these drugs as local anaesthetics for the production of surface anaesthesia.

TABLE III  
THE ANAESTHETIC POTENCY OF PP AND DP AS DETERMINED BY THE  
RABBIT'S CORNEA METHOD<sup>1</sup>

Drug	Duration of anaesthesia in minutes in rabbits numbered 1 to 9										Efficiency ratio
	1	2	3	4	5	6	7	8	9	Mean	
PP 0.25%	10	11	23	14	19	17	—	—	—	16	2
DP 1%	14	—	—	—	17	16	17	19	15	16	1/2
Cocaine 0.5%	10	10	23	13	15	17	17	21	18	15	1

*Intradermal weal method*—The method used was to inject 0.1 ml. of a sterilized solution intracutaneously on the flexor surface of the forearm. The duration of anaesthesia was determined by the time during which there was a loss of sensation of the injected area to pin pricks. Table IV shows the results obtained on three subjects; it will be seen that PP and DP were 12.5 and 5 times as potent as procaine respectively. Since the toxicities of PP and DP are

about six and four times that of procaine respectively, these results indicate that PP and DP may be of clinical value as local anaesthetics for the production of conduction anaesthesia

TABLE IV  
THE ANAESTHETIC POTENCY OF PP AND DP AS DETERMINED  
BY THE INTRADERMAL METHOD

Drug	Duration of anaesthesia in minutes in different subjects				Efficiency ratio
	Ho	Fan	Hsia	Mean	
PP 0.02%	11	12	14	12.7	12.5
DP 0.05%	13	11	10	11.3	5.0
Procaine 0.25%	10	11	9	10.0	1.0

#### ACKNOWLEDGMENTS

We are obliged to Mr M J Chang, of the New Asia Pharmaceutical Research Institute, for microanalyses, and to the China Foundation for the Promotion of Education and Culture for a research grant awarded to one of us (Y T H)

#### SUMMARY

Two new phenyl urethanes, namely sym bis-(diethylamino)isopropyl phenylurethane and sym dipiperidino-isopropyl phenylurethane, were prepared. The dihydrochlorides of both exhibited marked local anaesthetic activity when tested by the rabbit's cornea and the human intradermal weal methods. In view of their activities and toxicities relative to cocaine and procaine they are considered worthy of clinical trial.

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## THE ANTIHISTAMINE SUBSTANCE 2786 R P

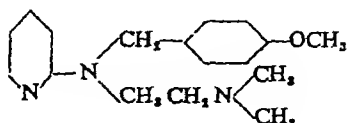
BY

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(Received Oct. 11 1946)

Bovet and Walthert (1944) have described the properties of a substance which is known as 2786 R P, and called by them neoantergan, it is *N*-dimethylaminoethyl *N*-*p*-methoxybenzyl- $\alpha$ -aminopyridine



This substance powerfully inhibits the action of histamine in animals and has been used clinically with success in the treatment of chronic urticaria and other conditions like hay fever. Benadryl (dimethylaminoethylbenzhydriol ether hydrochloride) was introduced by Loew, Kaiser, and Moore (1945) a year later, and Dr H O Schild informs us that when tested on the guinea-pig ileum in comparison with benadryl, neoantergan is 7 to 18 times more powerful against histamine, depending on the length of contact with the ileum, and 60 to 70 times less powerful against acetylcholine.

We have examined neoantergan by several of the methods used by Bovet and Walthert, and have confirmed their main results. We have also examined neoantergan by several other methods which will now be described.

### EXPERIMENTAL OBSERVATIONS

*Action on isolated auricles*—Very little stress was given by Dale and Laidlaw (1910), in their original description of the properties of histamine, to the stimulant action of histamine on cardiac tissue. The stimulant action was first demonstrated by Gunn (1926). It is not, however, nearly so striking in the isolated heart perfused by Langendorff's method as when the auricles of the rabbit heart are dissected and suspended in a bath of well-oxygenated Ringer's solution at 28° C. Oxygenation is provided by a gas distribution tube at the bottom of the vessel. In these circumstances the addition of histamine in such amount that

the concentration of base is 1 in 1 million causes a large augmentation of the beat, the effect is seen twice in succession in Fig 1 Neoantergan was then



Fig 1—Isolated auricles of rabbit heart At A histamine was added to the bath (0.1 mg. to 100 ml.) Note the great increase of amplitude At B, 0.5 mg. neoantergan was added, after which the addition of histamine was without effect

added to the bath, and in its presence the addition of histamine was without effect.

*Action on the driven auricles*—Dawes (1946) has recently described a preparation to measure the action of quinidine on cardiac tissue. The isolated auricles of the rabbit are arranged in contact with electrodes so that they can be made to beat at an imposed rate. A maximum rate can be determined beyond which they cannot follow the stimuli applied, and this maximum rate is reduced when the auricles are exposed for a given length of time to a given concentration of quinidine, or of a substance having a similar action. We have tested neoantergan by this method, and have found that it is approximately twice as active as quinidine. Table I gives the results obtained in one of the experiments. A comparison was undertaken of the toxicity of neoantergan for mice with that of quinidine, both substances being given by intraperitoneal injection. It was found that neoantergan was approximately twice as toxic as quinidine, the LD<sub>50</sub> of neoantergan being approximately 120 mg per kg, whereas that of quinidine was approximately 225 mg per kg (About 30 mice were used for each substance). Neoantergan is thus of equal value to quinidine for its action on the heart, it is twice as active, but twice as toxic. It should be noted that

TABLE I  
COMPARISON OF NEOANTERGAN WITH QUINIDINE ON THE ISOLATED AURICLES OF THE RABBIT

Substance	Dose in mg. in 100 ml. bath	Percentage reduction in maximal rate
Quinidine	0.25	7.9
	0.5	17.2
Neoantergan	0.125	8.3
	0.25	15.3

the figure for the LD<sub>50</sub> of quinidine by intraperitoneal injection differs from that given by Dawes (1946). Dawes's figure was 135 mg per kg. The difference between our figure and that of Dawes serves to illustrate the point that figures for toxicity have no general validity, since they can vary by as much as 100 per cent in the same laboratory at different times. Such figures are only of use for comparing two substances at the same time (compare Burn and Greville, 1931).

*Action on coronary flow*—Histamine is well known to produce a dilatation of the coronary vessels of the cat, and since histamine is here relaxing smooth muscle we were interested to examine the action of neoantergan. The Langendorff preparation was set up, the heart being perfused with Ringer's solution at 37° C and the coronary flow was measured as the outflow from the

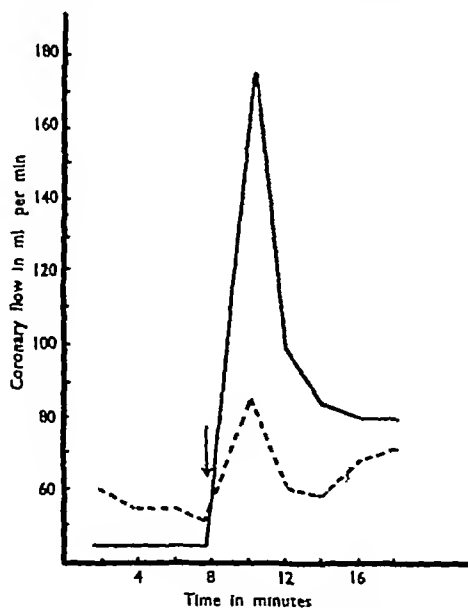


FIG 2—Abscissae: time in min. Ordinates: coronary flow in ml per min recorded in the heart-lung preparation of the dog. At the arrow 0.1 mg histamine was injected into the sup. vena cava with the effect shown in the continuous line. Later 0.1 mg neoantergan was injected followed 1 min. later by 0.1 mg. histamine. The effect of the histamine was that shown in the broken line.

heart. The results in different experiments were similar, and the following is an example. The injection of 25  $\mu\text{g}$  histamine caused an increase in coronary flow from 44 ml per min to 120 ml per min. When this effect had finally passed off in about 6 min, neoantergan was injected in a dose of 25  $\mu\text{g}$  and followed by 25  $\mu\text{g}$  histamine. The coronary flow increased to 6 ml per min only, and returned in 2 min to the previous rate.

A similar experiment was carried out in the heart-lung preparation of the dog, in which the coronary flow was recorded by inserting a Morawitz cannula in the coronary sinus. The result is shown in Fig 2. These experiments show that neoantergan reduces the effect of histamine in relaxing the smooth muscle of the coronary arteries.

*Action on blood vessels*—The vessels of the rabbit's ear perfused with Ringer's solution by the method of Gaddum and Kwiatkowski (1938) are constricted by small doses of histamine. We have found that this constrictor action is extremely sensitive to the presence of neoantergan and disappears when very small amounts are injected. Fig 3 shows that the constrictor action of 0.4  $\mu\text{g}$

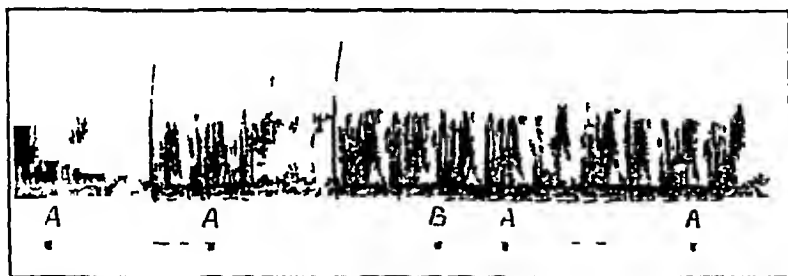


FIG 3—Perfusion of rabbit's ear with Ringer's solution. outflow recorded by Gaddum's drop-timer. At A, 0.4  $\mu\text{g}$  histamine injected. At B, 0.01  $\mu\text{g}$  neoantergan injected.

histamine was almost abolished by the previous injection of 0.01  $\mu\text{g}$  neoantergan, and we have observed that a significant reduction was produced by 0.001  $\mu\text{g}$  neoantergan.

In experiments in which blood flowed through the vessels, the effect of neoantergan on histamine was much less. In the hind-leg of the dog perfused by a pump, the vasodilator action of 3–5  $\mu\text{g}$  histamine was in two experiments greatly diminished and in a third little affected by the previous injection of 10  $\mu\text{g}$  neoantergan. In the cat anaesthetized with chloralose, when the spleen volume and the blood pressure were recorded, the intravenous injection of neoantergan abolished the action of histamine on the spleen, but not the depressor action on the blood pressure, even when a dose as large as 10 mg was injected (See Fig 4). On the other hand, in cats anaesthetized with ether, the injection of 2.5 mg neoantergan greatly diminished the action of histamine on the blood pressure, so that even 80  $\mu\text{g}$  produced less fall than 10  $\mu\text{g}$  previously.



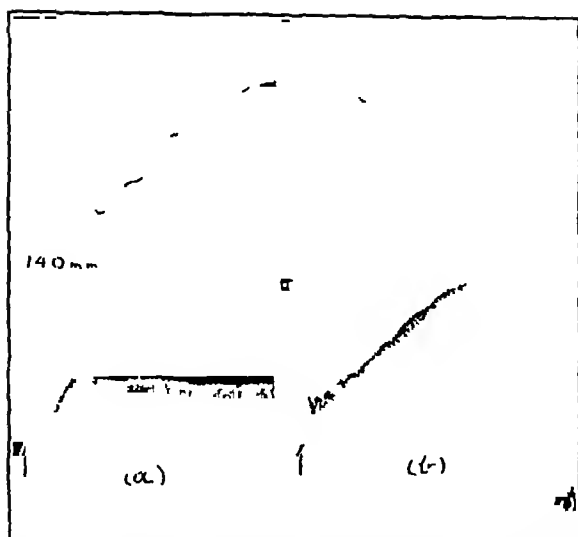


FIG 4—Cat, chloralose. Upper record is spleen volume, lower record is blood pressure. At the arrow  $10\text{ }\mu\text{g}$  histamine was injected intravenously. Between (a) and (b)  $2.5\text{ mg}$  neoantergan was injected. Note the abolition of the action on the spleen, while the blood pressure effect remains.

In the rabbit anaesthetized with urethane, the injection of  $5\text{ mg}$  neoantergan somewhat reduced the duration of the fall of blood pressure produced by  $0.25\text{ mg}$  histamine, but wholly abolished the stimulant action of this dose of histamine on the respiration.

*Action on the uterus*—The common description of histamine as a stimulant of smooth muscle not only neglects the inhibitory action of histamine on the smooth muscle of the coronary vessels of the cat and dog, but also the inhibitory action on the uterine muscle of the rat. We have already seen that the inhibitory action on the coronary vessels is reduced by neoantergan, we have failed, however, to observe that neoantergan, in concentrations which have no effect on the uterus, reduces the inhibition of the rat uterus by histamine. This result confirms Bovet and Walther (1944), but we do not confirm their statement that neoantergan has little antihistamine action on the guinea-pig uterus. Fig 5 illustrates this effect produced by neoantergan in a concentration of 1 in 500 million.

*Action as a spasmolytic*—Neoantergan is an antagonist of acetylcholine on the isolated rabbit intestine, though its effect is slight. The stimulant action of  $5\text{ }\mu\text{g}$  acetylcholine was reduced to less than half by the presence of  $0.5\text{ mg}$  neoantergan in a bath of  $100\text{ ml}$ . This atropine-like effect is feeble compared with its antihistamine action on guinea-pig ileum, in which tissue the stimulant action of  $1\text{ }\mu\text{g}$  histamine is reduced to less than half by  $0.1\text{ }\mu\text{g}$  neoantergan.

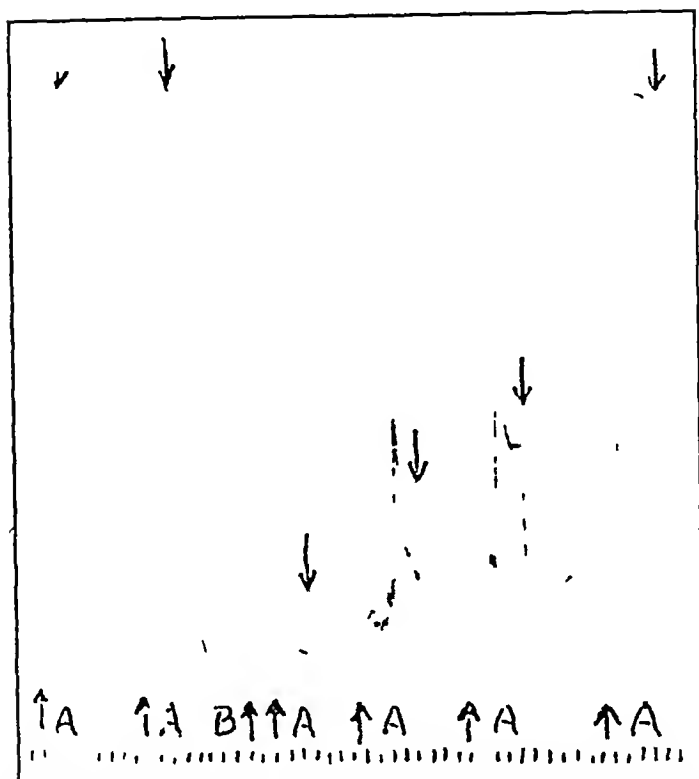


FIG 5—Isolated uterus of guinea-pig in bath of 100 ml. At A, 3  $\mu$ g. histamine added to the bath. At B, 0.2  $\mu$ g. neoantergan added. The bath was changed as soon as each contraction passed its maximum.

*Local anaesthetic action*—Since neoantergan has some atropine-like action, though very little, it was also tested for local anaesthetic action, using the guinea-pig intracutaneous test as described by Büllbring and Wajda (1945). After preliminary observations had shown that a local anaesthetic action was present, a comparison was made with procaine, in which solutions of neoantergan of strengths 0.06, 0.12, and 0.5 g/100 ml were compared with solutions of procaine of strengths 0.25, 0.5, and 1.0 g/100 ml. The mean result was that neoantergan is 3.1 times as active as procaine by this test.

*Analgesic action*—Neoantergan was tested for analgesic action by the method described by Thorp (1946). One group of rats was injected with pethidine, one group was injected with neoantergan, and one group was injected with saline. After determining the threshold for stimulation of the tail, the injections were given intravenously into the tail vein. Rats injected with 5.0 mg per kg pethidine then required a much stronger stimulus, the mean increase of threshold being 90.2 per cent.

The dose of neoantergan was as much as 40 per cent of the lethal dose, assuming that the LD<sub>50</sub> given by Bovet and Walther for mice is applicable to rats. This figure is 30 mg per kg, and the rats were given 12 mg per kg. The injection caused a slight but not significant increase in the threshold. Each group contained 5 rats, and the same general result was obtained in four separate experiments. Since in each of the last two experiments there was a rather greater rise of threshold in the rats injected with neoantergan than in those injected with saline, it is possible that neoantergan possesses some analgesic action, though, if so, it is feeble. There was, however, great variation among the rats in a group, and many observations would have been necessary to establish that the small rise in threshold was significant.

Further observations were made in which neoantergan was injected subcutaneously. Again there was evidence of a slight analgesic action in some rats when a dose of 30 mg/kg was given, it was not present in all. When doses of 100 mg/kg were given there was not only analgesia but a general narcotic effect. The mean rise of the threshold for stimulation of the tail was 80 per cent after one hour.

*Effect of daily administration on growth*—Since neoantergan is intended for clinical use it was tested to see whether daily administration exerted any deleterious effect. The maximum total daily dose for a man has been taken as 1.0 g, though it is usual to recommend patients to take 0.1 g three times a day.

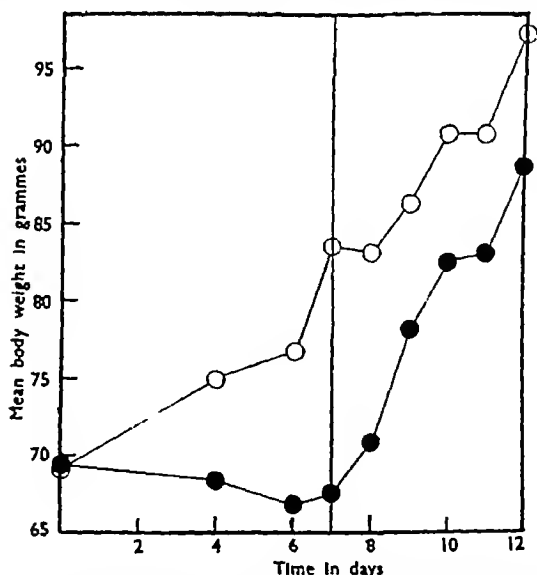


FIG 6—Abscissae, time in days, ordinates, mean body weight in grammes. White circles show the increase in body weight of 5 control rats, black circles show the body weight of 5 rats given 25 mg./kg. neoantergan twice daily by subcutaneous injection, for 7 days. After the seventh day the injections were stopped.

at first This dose is then doubled, and may be recommended four times a day, or again, the dose may be trebled A dose of 10 g for a man of 70 kg is equivalent to 14 mg/kg

The effect of giving a total daily dose of 50 mg/kg by subcutaneous injection was determined in a group of 5 young rats of 60–70 g The dose was divided into two parts, half being given in the morning, and half in the evening Fig 6 shows the arrest in growth when this dose was given for 7 days, and also shows that when the injections were stopped the rats at once began to grow at the same rate as the group of 5 control rats The cause of the arrest of growth may have been a decline in the amount of food eaten, though we do not know this, it was probably not due to a toxic effect on the liver or kidney, since growth restarted at once when injections stopped A second experiment was then carried out in which the total daily dose was 16 mg per kg In this experiment there was no effect on the growth of the 5 injected rats as compared with the 5 control rats Thus a dose equivalent to the maximum human therapeutic dose, when given in two injections daily for 11 days, did not affect the growth of young rats

### DISCUSSION

Perhaps the most striking method of demonstrating the effect of antihistamine substances is to use them to protect guinea-pigs against the action of histamine sprayed into the air from an atomizer We have used a box with a glass top similar to that described by Bovet and Walthert (1944), in which the guinea-pig is placed The box is about 12 in square and 6 in deep The nozzle of an atomizer is placed in a small hole in the floor of the box, and a solution of 1 or 2 per cent histamine (base) is sprayed into the box If a series of guinea-pigs is placed in the box, one at a time it is seen that they become asphyxiated and collapse in about 90 sec If they are promptly removed from the box, they recover The injection under the skin beforehand of 1 mg neoantergan gives complete and long-lasting protection against the histamine, and we have confirmed Bovet and Walthert's statement that as little as 0.1 mg/kg. gives considerable protection

The antihistamine action is also easy to demonstrate if a 1 in 1,000 solution of neoantergan is mixed with 1 in 1,000 histamine and a drop is put on the forearm If a prick is made through the drop, very little effect of histamine is seen

Dawes (1946) has pointed out that substances having a quinidine-like action possess other properties in addition Local anaesthetics, such as procaine and amethocaine have a quinidine-like action, and so have spasmolytics such as "syntropan" and "trasentm" Substances with an analgesic action like papaverine and pethidine also have a quinidine-like action We are now able to extend the list to include antihistamine substances for neoantergan has a quinidine-like action too It is becoming clearer that there is a large group of substances which possess in common all these different properties though in

very varying degrees Neoantergan is not only an antihistamine substance which possesses a quinidine-like action, but it is also a local anaesthetic, and it has a feeble action as an antagonist to acetylcholine on the intestine of the rabbit. It appears to have a slight action as an analgesic which is detectable by the test on the tail of the rat. The chemical relation of neoantergan to other substances with similar properties is not immediately obvious, but many other substances contain a chain resembling dimethylaminoethyl, and also a group corresponding to *p*-methoxybenzyl. In "benadryl" the linkage of the two is effected as an ether. In neoantergan the linkage is through  $\alpha$ -amino pyridine, this is certainly likely to be less toxic than a linkage through aniline, as in the earlier compound antergan (Halpern, 1942).

#### SUMMARY

1 The properties of the antihistamine substance 2786 R P, called by Bovet and Walthert neoantergan, have been examined, and the statements of these authors have, in the main, been confirmed. We observed, however, more antihistamine action on the guinea-pig uterus and less on the blood pressure of an animal anaesthetized with chloralose.

2 In addition the following effects have been observed. Neoantergan is a local anaesthetic three times as potent as procaine. It has a quinidine-like action on the auricle, twice as strong as that of quinidine. It abolishes the stimulant action of histamine on cardiac tissue, the dilator action on coronary vessels, and the constrictor action on the vessels of the rabbit ear. It has some analgesic action. When the maximum daily therapeutic dose calculated per kg is given to young rats daily for 11 days their growth rate is unaffected, though three times as much causes an arrest of growth.

Our thanks are due to Prof J H Burn for directing this work. The work was done during the tenure by one of us (J D P Graham) of an ICI fellowship awarded by the University of Glasgow. We are indebted to Dr D Bovet of the Institut Pasteur for a supply of the material. We also received some from Messrs May & Baker, Ltd.

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## THE TOXICITY OF ARSINE ADMINISTERED BY INTRAPERITONEAL INJECTION

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In a previous paper (Levy, 1946) accurate figures were given for the median lethal dose of arsine for mice. These figures were obtained by determining the arsenic in the entire carcasses after subjecting the animals to exposures to arsine which were known to produce 50 per cent mortality. Technical considerations prevented the application of this method for determining the lethal dose to larger animals. In experiments with different species of animal, it was found possible to administer arsine by intraperitoneal injection of the gas in admixture with hydrogen. Since the toxicity of arsine given in this way bore no direct relationship to its toxicity when inhaled, the experiments were discontinued at an early stage. It was considered that, although the number of animals used was small, the results obtained might possess some theoretical interest.

### EXPERIMENTAL RESULTS

Mixtures of arsine and hydrogen, prepared and assayed as previously described (Levy, 1946), were injected from a hypodermic syringe or gas burette. The arsine concentration in the mixture was usually about 10 mg./100 ml. Surviving animals were kept under observation for at least a fortnight after the injection or the last of a series of injections.

Figures for the mortality produced by single intraperitoneal injections of arsine into mice, rabbits, cats, and sheep are shown in Table I. The LD<sub>50</sub> of arsine administered in this way appears to have been approximately the same (2.5 mg. AsH<sub>3</sub>/kg) for all species of animal studied. Results obtained when arsine was injected repeatedly at intervals of 24 hours into mice and rabbits are summarized in Table II. For mice, the maximum tolerated daily dose appears to have been about 1.2 mg. AsH<sub>3</sub>/kg, approximately half the figure for the LD<sub>50</sub> by single injection, while for rabbits the corresponding figure was only 0.5 mg. AsH<sub>3</sub>/kg. It is strange that two injections of 1.0 mg./kg caused a greater percentage mortality in rabbits than a single injection of 2.0 mg./kg.

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\*Now Imperial Chemical Industries Research Fellow in Biochemistry

TABLE I  
LETHAL DOSE OF ARSINE ON INTRAPERITONEAL INJECTION

Animal	Dose, mg AsH <sub>3</sub> /kg.	Mortality	Approx LD50, mg. AsH <sub>3</sub> /kg.
Mouse	2.5 3.75 4.0 5.0	2/6 4/6 6/7 12/12	3.0
Rabbit	1.0 1.5 2.0 3.0 4.0 6.0	1/2 0/2 0/2 2/2 2/2 2/2	2.5
Cat	2.0 2.5 3.0 4.0 5.0	1/2 1/2 2/2 2/2 2/2	2.0-2.5
Sheep	2.0 4.0	0/2 2/2	3.0

TABLE II  
CUMULATION OF ARSINE INJECTED AT 24-HOUR INTERVALS

Daily dose, mg AsH <sub>3</sub> /kg.	Animals dying on daily injection		Animals surviving daily injection	
	No. of animals	No. of injections before death	No. of animals	Total no. of injections received
Mice				
0.3	—	—	4	8
0.6	—	—	8	10
1.2	1	5	7	10
1.5	1	8	3	14
1.5	4	3	8	3
2.0	1	2	2	2
2.0	5	3	—	—
2.5	2	1	1	2
2.5	5	2	—	—
Rabbits				
0.5	1	11	2	11
1.0	5	2	—	—
1.0	1	3	—	—
1.5	3	2	—	—

The clearance of arsine from mice injected with 4.0 mg/kg is shown in Fig. 1. At intervals after injection, the animals were killed and the arsenic in the carcasses was determined (Levy, 1943). Three mice were killed at each

time interval and the average figure for residual arsine is plotted in the graph. Half of the arsine injected was cleared in the first 24 hours, but most of the remainder was still present after another 18 hours. The clearance process was,

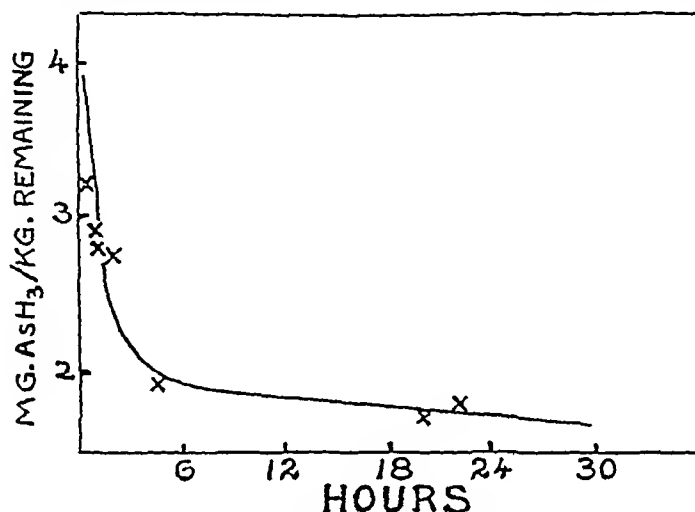


FIG 1—Clearance of arsine from mice after intraperitoneal injection 40 mg. AsH<sub>3</sub>/kg. injected

however, rapid enough to explain the tolerance by mice of daily injection with half the LD<sub>50</sub>

### DISCUSSION

From the results described above it appears unlikely that much arsine injected into the peritoneal cavity escaped fixation by the erythrocytes or the body tissues. Arsine administered in this way, however, is far from exhibiting its maximum toxicity. The toxicity for mice exposed to the gas (Levvy, 1946) was found to increase as the concentration inhaled increased, and with a concentration of 2.5 mg AsH<sub>3</sub>/litre in the atmosphere, the LD<sub>50</sub> was only 0.67 mg AsH<sub>3</sub>/kg. It did not approximate to the figure obtained when the gas was given intraperitoneally (2.5 mg/kg) until the atmospheric concentration had been reduced to 0.25 mg/litre.

In contact with blood, arsine is fixed by the erythrocytes, and undergoes reaction with the haemoglobin. When haemolysis ultimately occurs the arsenic-containing product or products of the reaction are liberated. Fixation of the arsine occurs so rapidly that it has been held until recently that all arsine entering the body must go through this process. Arsenical poisoning of body tissues was considered, on this view, to be due entirely to the product or products of the reaction in the red blood corpuscles. An arsenite is the most toxic compound likely to be produced in the reaction. The lethal dose of an arsenite administered



parenterally appears to be constant for all species of animal at about 5 mg As/kg (Fischl and Schlossberger, 1934). To explain the much lower figure observed for the LD<sub>50</sub> of arsine when it was inhaled in high concentration, it was suggested in the previous paper (Levy, 1946) that part of the gas inhaled escapes fixation by the erythrocytes long enough to be carried in physical solution in the plasma to vital organs and to produce a specific effect in them.

The fact that the LD<sub>50</sub> of arsine when it was given intraperitoneally was 2.5 mg/kg may indicate that the conditions of absorption from the peritoneal cavity are such that the fraction of the gas not fixed by the erythrocytes is the same as during inhalation of arsine from an atmosphere containing 0.25 mg/litre. If absorption of arsine from the peritoneal cavity is at all delayed, it is unlikely that much of the gas can escape fixation in the blood. In this case, the effects on body tissues must be due solely to the arsenic derivative liberated on haemolysis. This possibility is not incompatible with the belief that the compound formed in the blood is an arsenite, since haemolysis may contribute sufficiently to the toxicity for the whole animal to explain the smaller figure for the LD<sub>50</sub> on injection of arsine than of arsenite. The alternative explanation, that the figure observed for the LD<sub>50</sub> of arsine when it is administered intraperitoneally indicates that as a result of the reaction in the blood the gas is converted into some unknown compound (almost certainly trivalent—see Graham, Crawford, and Marrian, 1946) intermediate between arsine itself and arsenites in the severity of the effects it produces in the rest of the body, cannot be excluded.

#### SUMMARY

- 1 It was found possible to administer arsine quantitatively to animals by intraperitoneal injection of arsine-hydrogen gas mixtures.
- 2 The median lethal dose of arsine administered in this way was approximately 2.5 mg/kg for all species of animal studied.
- 3 From cumulation and excretion experiments it was concluded that half the dose injected into mice was cleared from the body in 24 hours. With rabbits, the clearance appeared to proceed more slowly.
- 4 Possible explanations are discussed of the fact that the median lethal dose of arsine in these experiments was much greater than the lowest figures observed when the gas was given by inhalation.

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